HEAVY METAL CONTAMINATION AND METALLOTHIONEIN mRNA LEVELS IN THE TISSUES OF TILAPIA

M. Phil. Thesis

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Abstract

The purpose of this research is to investigate the responsiveness of metallothionein (MT) gene expression in the tissues of tilapia (*Tilapia* spp.) following metal administrations. Tilapia is a euryhaline fish that exists worldwide and is a dominant species in local inland waters and estuarine regions. Intraperitoneal injections, aqueous exposures, and analyses of feral samples from a local river were performed.

In the present study, we investigated MT gene expression of tilapia exposed to heavy metal ions (copper, zinc and cadmium) through intraperitoneal injection and aqueous exposure. In metal injection tests, all tissues except kidney, injected with metals, indicated higher level of MT mRNA than injected with saline. Liver in tilapia injected with cadmium gave the highest induction of MT mRNA, however kidney in the zinc-injected tilapia showed higher induction level of MT mRNA. In aqueous exposure test, liver of tilapia exposed to low doses (50 μg/L) of cadmium and zinc showed high correlation between fold induction of MT mRNA over a 3 week period tested (r=0.813 for cadmium, 0.9783 for zinc). However, at 100 μg/L of cadmium and zinc in the liver of tilapia gave negative correlation. At high doses of zinc (0.4, 1, 2 mg/L) at week 1 and week 2 gave good correlation with hepatic MT mRNA (r=0.942 for week 1, 0.974 for week 2). Results from carp are also presented for comparisons.

Apart from laboratory studies, we also investigated tilapia MT mRNA from different sites (Tai Wai and Fo Tan) in Shing Mun River. In order to correlate the metal concentrations and MT mRNA levels of different tissues (liver, gill and muscle), Atomic Absorption Spectrophotometer and Northern Blot Analysis were employed to measure metal concentrations and MT mRNA levels respectively. There was no significant correlation in the tissues of feral tilapia from Tai Wai and Fo Tan.

Studies on tilapia MT gene sequences were also performed. The MT genes including 3 exons, 2 introns and 3' untranslated regions (610 bp) of tilapia from different sites (Marine Science Laboratory (MSL), Agriculture and Fisheries Department of Hong Kong Government (AFD) and Fo Tan) were amplified by PCR and cloned in pCR2 vector using TA cloning method. Comparing the MT gene sequences in tilapias, high homology (97%) was obtained indicating the MT genes are highly conserved in the genomes of tilapia.

摘要

金屬硫蛋白(Metallothionein,MT)是一種細胞內攝合重金屬的低份子量蛋白質。在魚類之中，牠們器官所含MT的水平和重金屬的含量是有極大關連。本研究是分析不同重金屬濃度對非洲鯽魚MTmRNA的表達。首先，重金屬腹腔注射的測試，表明非洲鯽魚不同器官的MTmRNA表達的程度。除了腎臟外，被重金屬注射的非洲鯽魚器官之MTmRNA水平是較被注射鹽水的爲高。特別在鎘（Cd）的注射中，肝臟的MTmRNA的水平是最高的。另外，非洲鯽魚暴露在不同濃度的重金屬水中，低濃度（50μg/L）的鎘（Cd）及鋅（Zn）顯示魚肝的MTmRNA水平與暴露週期的相關係數頗高（鎘（Cd）是0.813而鋅（Zn）是0.978）。另外，本研究也包括在曾經受到嚴重污染的城門河內（火炭和大圍）收集非洲鯽魚，並量度魚的器官內MTmRNA的水平及重金屬的含量。最後我們亦利用多聚酶鏈式反應（PCR）去擴增和克隆了不同來源（漁農處, *Tilapia aureus* x *Tilapia niloticus* ; 海洋科學研究中心, *Tilapia niloticus* ; 和火炭, *Tilapia mosambicca* 的非洲鯽魚MT的基因(610bp)發現牠們的MT基因是非常相似(97%)。

Abbreviations

List of Abbreviations

A	Adenosine
ARE	Antioxidant responsive element
BLE	Basal level element (of human MTIIA gene)
bp	Base pair(s)
C	Cytidine
Cd	Cadmium
Cu	Copper
Cr	Chromium
Ni	Nickel
Pb	Lead
DEPC	Diethyl pyrocarbonate
cDNA	Complementary deoxyribonucleic acid
C-terminal	Carboxyl-terminal of a protein
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
G	Guanosine
GRE	Glucocorticoid responsive element
IPTG	Isopropanyl-β-D-thiogalactopyranoside
kb	Kilobase(s)
μ	Micro-(10^{-6})
M	Molarity
m	Milli-(10^{-3})
MRE	Metal responsive element
mRNA	Messenger ribonucleic acid
n	Nano-(10^{-9})
N-terminal	Amino-terminal of a protein
NF-1L6	Nuclear factor interleukin 6

O.D.	Optical Density
PCs	Phytochelatins and cadystins
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SSC	Standard saline citrate
T	Thymidine
TAE	Tris-acetate-EDTA
TBE	Tris-boric acid-EDTA
TE	Tris-EDTA
TRE	Tumor responsive element
Tris	Hydroxymethyl aminomethane
UV	Ultraviolet
Xgal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
Zn	Zinc

Abbreviation Table for Amino Acids

Amino Acid	Three-letter	One-letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glyine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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Chapter 1 Introduction

1.1 Metallothionein

1.1.1 Classification of MT

Metallothionein (MT) was discovered as a result of investigating a biological role of cadmium in equine (Margoshes *et al.*,1957). Although extensive studies were done in the past 40 years, the function of this low molecular weight protein is still not well understood. MT has been found in a wide variety of eukaryotes (yeasts, echinoderms, crustaceans, insects, all vertebrates, and plants) and some cyanobacteria (Andrews,1990; Rauser,1990). The broad definition has made it useful to classify MT into three classes according to Shaw III *et al.* (1992).

Class I : Mammalian MT with 20 cysteines and closely related MT such as 18-cysteine MT of crustaceans and alignment with cysteine is possible.

Class II : Polypeptides, such as yeast MT, with locations of cysteines distantly related to mammalian MT.

Class III : Enzymatically synthesized peptides having a general formula:

$$\gamma\text{-(Glu-Cyc)}_n\text{Gly}$$

or poly(γ-glutamyl-cysteinyl)glycines, where n varies from 2 to 8 or higher, although 2 to 5 are the most common. They are variously known as phytochelatins (PCs) and cadystins and can be found in many plants and single cell organisms.

1.1.2 Structure of MT

Vertebrate MT is a low molecular weight Class I MT protein (6000-7000 daltons) which only consists of 60 to 68 amino acid residues without aromatic amino acid and histidine residues. The remarkable feature is its high cysteine residues (33%) in its amino acids which do not form disulphide bridges and are characteristically arranged in Cys-Xaa-Cys or Cys-Xaa-Xaa-Cys motifs, Xaa denoting any amino acid except for cysteine (Kojima,1991; Kagi *et al.*,1988; Vallee,1995).

According to Nuclear Magnetic Resonance spectroscopic and X-ray crystallography studies, the MT polypeptides form 2 globular domains, α-(C-terminal half) and β-domain (at the N-terminal half) connected by amino acid residues 30 and 31 as a hinge region of prolate ellipsoid (Nielson *et al.*, 1984). The α-domain and β-domain contain 11 and 9 cysteine residues respectively. They can bind metal ions in 2 distinct configurations: M_7 and M_{12} state. The distribution of divalent metal ions such as cadmium in M_7 state is $M_4\alpha$ and $M_3\beta$, and the metal ions are coordinated tetrahedrally, whereas the monovalent metal ions such as copper are bound in polynuclear clusters with a distribution of $M_6\alpha$ and $M_6\beta$ and are coordinated trigonally (Vasak, 1983; Kirk *et al.*, 1985).

The hinge region is located in the center of the α-domain and β-domain, which bind to metal ions independently. These domains possess different binding affinity to metal ions such as zinc and cadmium, which fill completely to the α-domain first and then bind to the β-domain. In contrast, copper binds cooperatively to the β-domain first (Nielson *et al.*,1984). However, analysis of MT with interdomain hinge expanded by insertion mutagenesis indicated that the level of metal binding activity declined with increased length of the hinge insert (Rhee *et al.*,1990). A comparison of bond strength of the metal-MT (Petering *et al.*,1990) is shown as follows:

Hg>Cu>Cd>Zn;

Pt>Cu;

Au~Cd.

Apart from chicken and many teleost species (e.g. flounder and plaice), more than 1 isoform have been found in Class I MT. For instances, the two common MT isoforms in mammals found are MT-I and MT-II (61 amino acid residues) which are different in their mobilities on the anion-exchange chromatography due to glutamic acid instead of glycine at position 10 of polypeptides in MT-II (Olsson *et al.*,1993). They are expressed and regulated coordinately in the tissues of mouse and are thought to perform similar functions (Kagi *et al.*,1987). However, MT-A and MT-B are assigned in some cases such as sea urchins and trout, for those amino acid sequences are derived from the complementary Deoxyribonucleotide (cDNA) without knowledge of the corresponding protein elution patterns.

Two other isoforms, MT-III and MT-IV were found in human and mouse respectively (Palmiter *et al.*,1992; Quaife *et al.*,1994). In human, MTIII contains 2 additional insertions: a single threonine residue at position 5 and a six-residue insert at residue 55, whereas MT-IV has one insertion at the N-terminal. Unlike MT-I and MT-II, MT-III and MT-IV are not found in all tissues. They are not likely to be metal inducible and are restricted to exist in specific tissues such as brain and differentiated stratified squamous epithelia cells respectively (Palmiter *et al.*,1992; Quaife *et al.*,1994). In fact, different isoforms of MT present in different species and are regulated independently. This is possible to fulfill unique biological roles in different species.

1.1.3 Structure of MT genes

The MT gene family is more complex than expected because multiple MT genes are present for MT synthesis. In human, 14 MT genes are clustered in a locus on chromosome 16 (Stennard *et al.*,1994); whereas in mouse, 4 MT genes are closely linked on chromosome 8 in a 45 kbp region and all are transcribed in the same direction (Palmiter *et al.*,1993) (Fig. 1.1). Wests (1990) reported that in human, there was a cluster of 13 closely related MT-I isogenes and one MT-IIA gene on the region of chromosome 16 (Palmiter *et al.*,1993). MT-III was located in human chromosome 16 and mouse chromosome 8 (Palmiter *et al.*,1992).

In human, however, only a few MT genes (namely MT-IE, MT-IH, MT-IIA and MT-III) are expressed to produce proteins. Other genes might be pseudogenes, which are not involved in the formation particular proteins (Schmidt *et al.*,1985). The pseudogenes might have early termination codon, or lack TATA-like sequence in the upstream region although they retain introns (Schmidt *et al.*,1985). Evolutionary considerations revealed the consensus DNA sequences in both the coding and regulatory regions of these MT genes (Schmidt *et al.*,1985).

The function of the multiple human MT genes is still unclear, but it is possible that these MT genes give responses to different metal ions either by changing the functional parameters of MT isoforms such as binding affinities for different metal ions or by differential expression of each isoform (Stennard *et al.*,1994). Indeed, there is now considerable evidence for inducer-specific (Cavigelli *et al.*,1993; Sadhu *et al.*,1988) and cell-type specific expression of each MT isoform (Varshney *et al.*,1986; Jahroudi *et al.*,1990; Heguy *et al.*,1986).

Vertebrate MT genes have a remarkable conservation of tripartite structure. Three exons are disrupted by 2 introns that vary substantially in length and nucleotide sequence but the sites of interruption are conserved from trout to human (Gedamu *et al.*,1993). The first exon contains the 5' untranslated region and encodes the first few amino acid residues of the β-domain. The second and third exons encode the remainder of the β-domain and the α-domain respectively. The end of exon 2 is also conserved in coding serine residue, which acts as a hinge for the two globular domains (Fig.1.2).

1.1.4 Function of MT

Since its discovery in 1957, MT has remained a protein in search of a function. After 40 years of extensive studies, MT is believed to be involved in (1) homeostasis of essential metals, (2) metal detoxification, (3) scavenging free radicals. In the MT-I and MT-II knock-out mice, MT is likely to detoxify heavy metals such as cadmium by sequestering metal ions and elimination of metals from organs like liver and kidney (Liu *et al.*,1996). As a result, the animals with MT expression can tolerate a higher concentration of metal ions sequestered by MT.

The remarkable feature of MT is its inducibility following metal ion administrations. According to the results of Kramer *et al.* (1996), cadmium and zinc treatment could induce MT or MT mRNA in mouse astrocyte primary cultures. In addition, Tort *et al.* (1996) reported that about three times higher levels of liver MT in fish exposed to cadmium as compared to the untreated. In *in vivo* experiment, injection of arsenic in rat enhanced rat hepatic MT synthesis. These data indicated that a variety of metal ions might increase MT expression either directly by inducing MT mRNA or indirectly by altering post-transcriptional event (Albores *et al.*,1992).

Apart from these, MT may control the endogenous level of essential metals such as copper and zinc, which are needed for maintaining normal metabolism. Olsson *et al.* (1990) reported the high endogenous hepatic levels of zinc- and copper-bound MT in the developing embryo of rainbow trout. Studies on winter flounder showed that hepatic zinc and copper levels varied with season and that zinc was incorporated into low-molecular weight proteins during the summer feeding season (Shears *et al.*,1985). These changes correlated to the period of sexual maturation in winter flounder (Fletcher *et al.*,1978). However, the results from Cu-thionein knock-out yeast and MT-I and MT-II knock-out mice strongly suggested that MTs are not essential for developmental processes (Masters *et al.*,1994). It is believed that MT can serve as an intracellular storage protein for these essential metals during perinatal development.

MT in intestine is thought to be an important site for the regulation of mammalian and avian zinc absorption. Cousins *et al.* (1978) demonstrated that parenteral injections of zinc ions and diets high in zinc ions resulted in the synthesis of MT in the intestinal mucosa of rats. In contrast, studies conducted to assess the involvement of MT in gastrointestinal zinc uptake in flounder indicated that its presence in the intestinal cytosol was not associated with any enhancement or depression of zinc uptake (Shears *et al.*,1984).

The ability of MT to scavenge free radicals has been intensively studied in recent years. The administration of superoxide radical generator caused an increase in MT concentration in the lung and liver of rats (Sato *et al.*,1989; 1991). The induction of MT by cytokines in the liver exerted an antioxidative role during acute phase response, therefore preventing tissues from injury by oxidative stress. Apart from functions mentioned, MT seemed to play a role in cancer chemotherapy (Cherian *et al.*,1994) and protection of mammalian cells against UV irradiation and alkylating agents (Hanada *et al.*,1991; Hanada *et al.*,1993). The discovery of MT-III and MT-IV in mouse and human might give

a insight on the function of MT. MT-III is a growth inhibitory factor in human brain tissue (Uchida *et al.*,1991) and is down-regulated in Alzheimer's disease (Tsuji *et al.*,1992), whereas MT-IV plays a role in development of differentiating cells of stratified squamous epithelia (Quaife *et al.*,1994).

1.1.5 Regulation of MT Expression

MT gene expression can be stimulated by various factors including metals or non-metallic compounds such as glucocorticoid hormones and UV irradiation (Kagi,1991). Among these inducers, heavy metals are the most potent and extensively investigated.

The fold-inductions of MT mRNA and MT vary greatly against different metal ions in *in vivo* or *in vitro* experiments. Durnam *et al.* (1981) reported that the mouse tissues (liver, kidney and pancreas) accumulated high levels of MT transcripts in response to metal ions; Compere *et al.* (1981) presented that some mouse lymphoid cell lines were resistant to metal induction of the MT genes. In addition, metal ions differ in efficacy of induction of MT. Mouse MT-I and MT-II gave higher responsiveness to cadmium than zinc and copper (Cadmium > Zinc > Copper) (Yagle *et al.*,1988).

The presence of Metal Responsive Elements (MREs) which has a consensus sequence as '-TGCRCnC-3' in the regulatory sequences of MT genes represented evidence for specific metal-induced transcription of MT (Thiele,1992). There are multiple copies of MRE in the promoter region of Class-I MT genes (Hamer,1986). For instance, the mouse MT-I gene has five functional MREs within the 150 bp flanking the transcription start site, but they are not functionally equivalent (Stuart *et al.*,1985; Searle,1990). Studies to date indicated a single MRE of the rainbow trout MT promoter could confer metal-inducible transcription to a reporter gene when cloned as tandemly repeated sequences upstream of the reporter gene's TATA box (Olsson *et al.*,1996).

Although other regulatory elements such as Sp1, AP1 and AP2, are also presented in the promoter regions of many MT genes, metal-inducible transcription of MT is only mediated by MREs (Mueller *et al.*,1988).

The metal regulatory factor MTF-1 in mouse and human were cloned and found to have six zinc-fingers similar to RNA polymerase III transcription factor TFIIIA (Cys_2His_2) at the N-terminal (Radtke *et al.*,1993; Brugnera *et al.*,1994). The zinc finger factors bind specifically to heavy metal-responsive DNA sequence elements in MT gene promoters and is essential for basal and heavy metal-induced transcription. Radtke *et al.* (1995) reported that there were three putative activation domains (namely an acidic domain, a proline-rich domain and a domain rich in serine and threonine at the C-terminal) which are downstream of the zinc finger region. The proline-rich and acidic activation domains also contribute to metal inducibility of target genes (Radtke *et al.*,1995). It was proposed that MTF-1 was a candidate transcription factor for interaction with MREs, but it is not clear how it is regulated by metal ions (Radtke *et al.*,1993).

Other metal ions such as cadmium, copper and mercury did not activate the MTF directly (Minichiello *et al.*,1994; Seguin,1991; Koizumi *et al.*,1992; Otsuka *et al.*,1993). In Fig.1.3, cadmium, copper and mercury with high affinities for ligands displaced zinc from other zinc-binding sites through metal-metal exchange reaction. The displaced zinc was then available for binding to the inhibitor, releasing the transcriptional factor from inhibition and initiating MT expression. The zinc ions released from inhibitor were chelated with newly induced MT and then displaced by cadmium discharged from binding sites of ligand (Roesijadi,1996). The actual mechanism of the activation of MT gene transcription, especially in the non-mammalian species, deserves of more investigation.

Apart from MREs, some other cis-acting elements such as some hormone and growth factor responsive elements are located in MT promoter such as glucocorticoid responsive element, GRE, in human MT-I promoter; and nuclear factor interleukin 6, NF-IL6, in rainbow trout MT-A promoter (Olsson *et al.*,1995). Furthermore, antioxidant responsive element (ARE) is found in the MT promoter regions in organisms from *C.elegans* to human (Dalton *et al.*,1994). In addition, Beach *et al.* (1981) and Price-Haughey *et al.* (1987) reported that the transcription of MT genes in a number of cell lines, such as mouse and rainbow trout respectively, were decreased by cytosine methylation, whereas demethylation could rescue the MT gene from inhibition (Lieberman *et al.*,1983).

1.1.6 Fish MT

After the first reported amino acid sequence of a teleost MT was a partial N-terminal and C-terminal sequence obtained from plaice (*Pleuronectes platessa*) MT (Overnell *et al.*,1981), many teleost's MT sequences were found as shown in Fig.1.4. Alignment of the deduced amino acid sequences of fish MT showed the cysteine residues were highly conserved and all the other sequenced teleost MT mRNAs contain 60 amino acids. However, MT-A in rainbow trout consists of 61 amino acids with an additional residue in the middle hinge region (alanine), (Bonham *et al.*,1987; Chan *et al.*,1989; George *et al.*,1989; Kille *et al.*,1991). Only one isoform was found in marine flatfish such as plaice, winter flounder and in some freshwater fish such as stone loach and pike. Another fish species such as carp and perch showed 2 MT isoforms (George *et al.*,1989).

Different from mammalian MT, teleost MT has only 3 amino acid residues before the first cysteine residues. The amino-terminal has been indicated as the primary antigenic site on MT (Kay *et al.*,1991). This may explain the low cross-reactivity of mammalian MT antibodies with teleost MTs and *vice versa* (Kay *et al.*,1991; Norey *et al.*,1990).

Teleost and mammalian MT genes have some similar features: (1) tripartite gene structure; (2) conservation of cysteine residues; (3) presence of multiple MREs in their 5'flanking regions. Like mammalian and yeast MTs, teleost MT gene promoters such as rainbow trout MT-A (Olsson *et al.*,1995; Hong *et al.*,1992) and MT-B (Zafarullah *et al.*,1988), pike and stone loach (Zafarullah *et al.*,1988) and a carp MT gene (Chan,1998) were studied in detail. A 250 bp sequence of rainbow trout MT-B promoter region, upstream of the transcription initiation site, was sequenced and subjected to functional analysis (Imber *et al.*,1989; Olsson *et al.*,1989). The promoter region of rainbow trout MT-A and MT-B contains a TATAA box and two metal responsive elements MRE-A and MRE-B. Additional MREs have been located at the distal regions of the promoters in rainbow trout MT-A, pike MT and stone loach MT (Olsson *et al.*,1993).

Unlike the human MT promoter regions, no GC boxes, Sp1 sites, AP1 sites, glucocorticoid regulatory elements (GRE) or tumor responsive elements (TRE) have yet been identified in the rainbow trout promoters (Olsson *et al.*,1993). However, carp MT gene promoter contains 2 Sp1 sites and 1 AP1 site in addition to 5 MREs (Chan,1998).

Teleost MT genes give different induction levels in response to different metals. Injection of cadmium to winter flounder gave higher induction MT mRNA in the liver (Chan *et al.*,1987). George *et al.* (1996) reported that different levels of MT synthesis were stimulated in the tissues (liver> kidney>gills) of marine flatfish. Zafarullah *et al.* (1989) indicated that teleosts were more sensitive to zinc, following by cadmium and then copper. The MT inducibility against cadmium in flounder is greater than other metals such as copper, lead and zinc (Chan *et al.*,1989).

MT gene expression is also dependent on developmental status, seasons and sex. In rainbow trout, MT mRNA increased at gastrulation, and then reached a peak at hatch, a stage that most MT were bound with copper and zinc (Olsson *et al.*,1990).

1.1.7 Aims and Rationale of the Present Study

In Hong Kong, there are some 600 rivers, streams and open nullahs located mostly in the New Territories. Most of these watercourses are typically short streams and have fast flowing upper reaches with rocky substrates, and sluggish flowing lower reaches. These watercourses provide a valuable resource to human life. However, some of these watercourses, particular in their lower reaches, have been polluted by anthropogenic activities such as illegal discharge of industrial effluents and domestic wastes due to industrial development and urbanization in rural areas. Since the water quality was deteriorated, it raised the public concerns on preventing the deterioration of river pollution. The government had implemented the Water Pollution Control Ordinance (WPCO) and the Water Disposal (Liverstock Waste) Regulation to control the water quality since 1987 (Environmental Protection Department,1995).

Fish is ecologically important as it is one of the most widely distributed organisms in the aquatic environment and about 50% of the vertebrate species are fish. Fish species is very sensitive to various environmental pollutants. The health and physiology of the fish species as well as the fish population may be severely damaged by environmental pollutants. In addition, as fish is a source of food of human and most of the pollutants are persistent in nature, human would consume considerable amount of pollutants via food chains and therefore human health would also be indirectly affected.

Tilapias are one of the cichlid fishes and are divided taxonomically on the basis of their brooding behaviour (Trewavas,1983). The genus *Tilapia*, *Sarotherodon* and *Oreochromis* , the members belong to Tilapias, are all Old World species originating in Africa. The genus *Tilapia* is substrate spawners , whereas the genus *Sarotherodon* comprises both paternal and biparental mouthbrooders. In contrast, the genus *Oreochromis* is solely maternal

mouthbrooders (McAndrew *et al.*,1984) which is widely distributed in local rivers and intertidal zones. Moreover, it is also commonly found in the freshwater pond, streams and estuarine region in Hong Kong. *Oreochromis* spp. is a tropical and herbivorous fish species, which is less tolerant to low water temperature (lethal below 12°C) and feeds on plant, algae and dead plant. Occasionally, it consumes some small crustaceans like small shrimps and worms. According to the American Fisheries Society, all three names of tilapia, *Sarotherodon* and *Oreochromis* had been unified to tilapia (American Fisheries Society, 1991)

In the present study, Tilapia spp. is chosen because it is dominant in local watercourse. Furthermore, even the aquatic environment is highly polluted, the species can also be found. Therefore, it seems to be a pollutant resistant species. In addition, it is a fast growing species and large enough to provide sufficient amount of tissues for different analyses. Finally, there is little study on MT gene expression in Tilapia spp. from local water.

The aims of the present study are as follows:

1. To determine the inducibilities of MT mRNA in the tissues (liver and gills) of tilapias injected with or exposed to metals (copper, cadmium and zinc).

2. To investigate metal concentrations (copper, cadmium, chromium, lead, nickel and zinc) in the tissues (liver, gills and muscle) of tilapias caught from Shing Mun River.

3. To study the MT mRNA levels in the tissues (liver and gills) of tilapias harvested from Shing Mun River and their correlation with concentrations of various metals.

4. To obtain the sequence of MT gene in the tilapias from different sites (Fo Tan, Marine Science Laboratory and Agriculture and Fisheries Department).

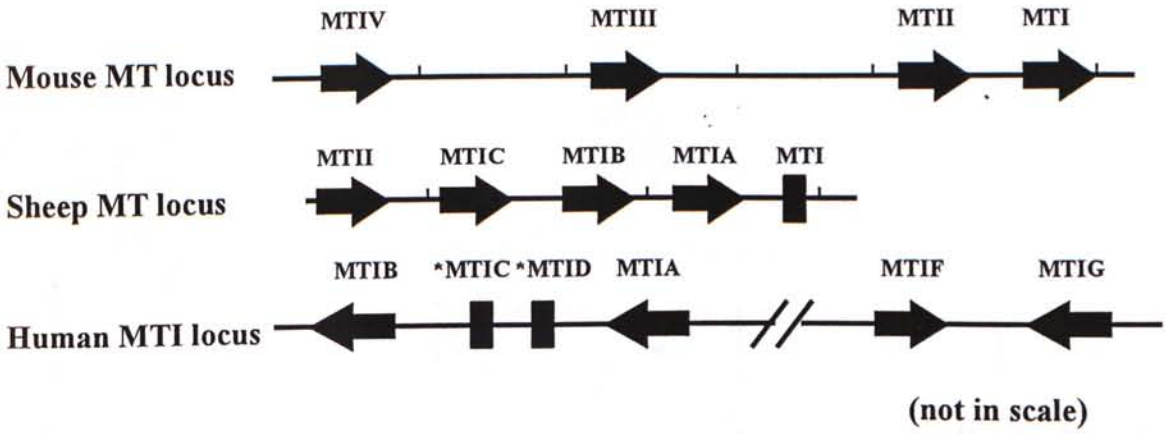


Fig. 1.1 MT loci in different mammalian species * indicates pseudogene **(Adapted from Andrews, 1990; Peterson *et al.*, 1988).**

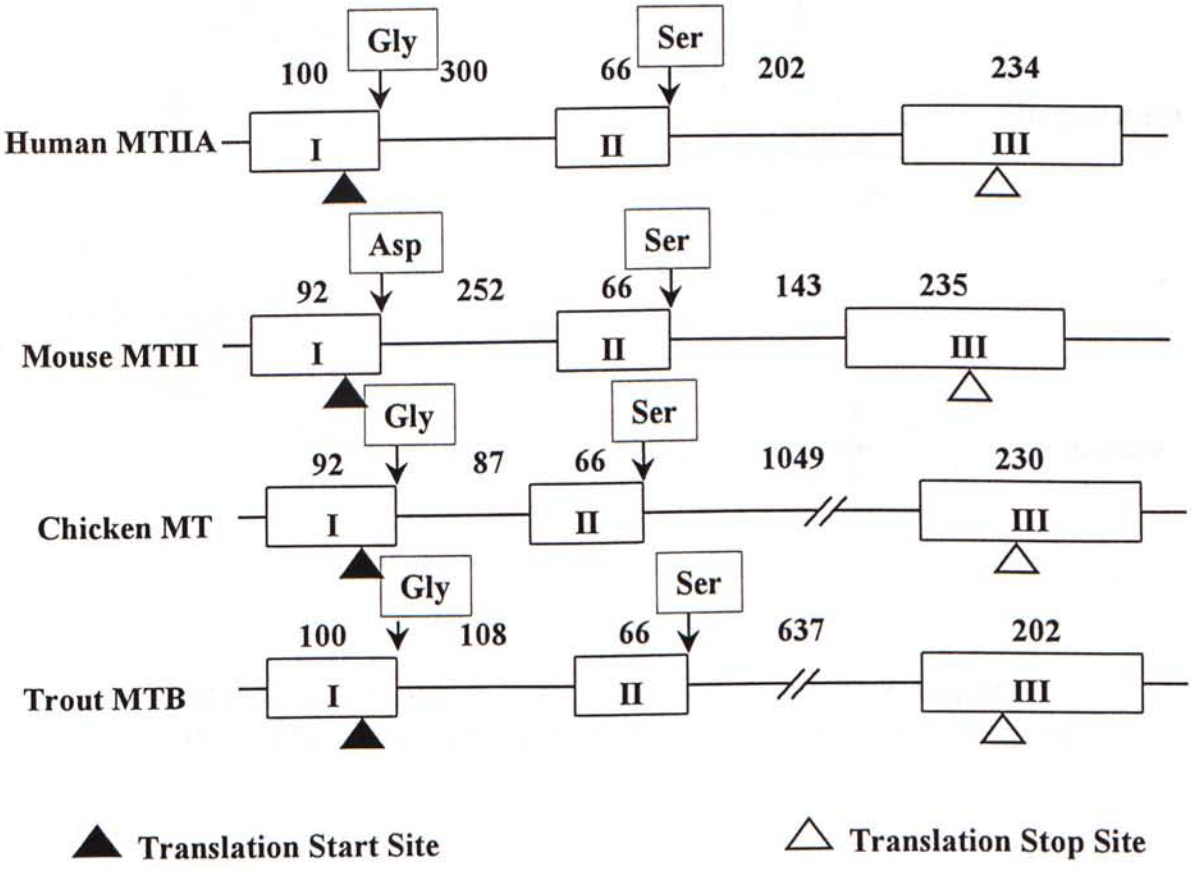
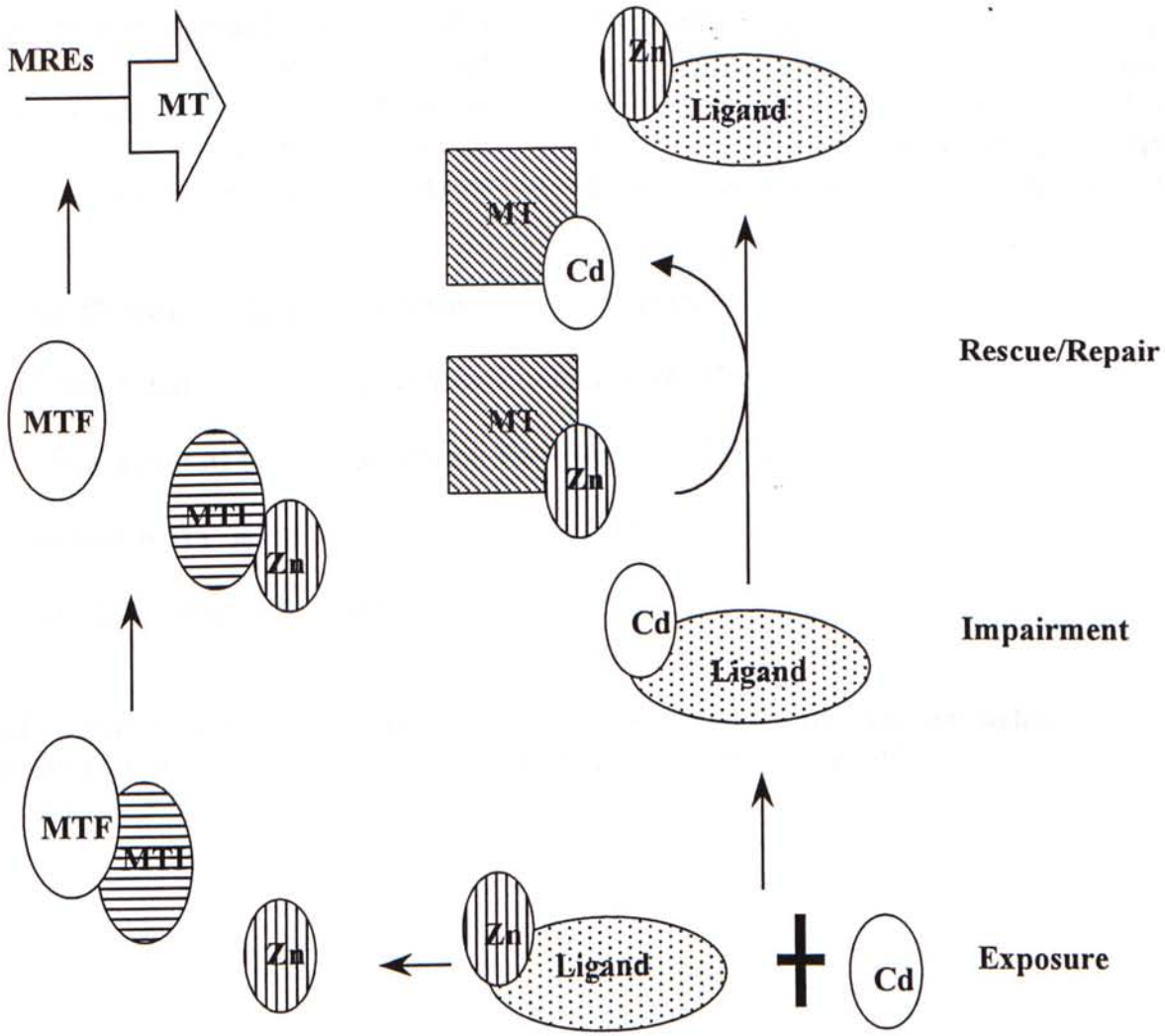


Fig.1.2 Tripartite structures of MT Genes. The numbers indicate the size of MT introns and exons in base pairs. The amino acids interrupted by the placement of the introns are also indicated as↓ (Adapted from Andrews, 1990).



Key : MT, Metallothionein; MRE, Metal Regulatory Element; MTF, Metal Transcription Factor; MTI, Metal Transcription Inhibitor.

Fig.1.3 Model for coupled MT induction and rescue of target ligands compromised by inappropriate metal binding, for example by Cd (Adapted from a review by Roesijadi *et al.*, 1996).

```
10        20        30        40        50        60
MDPCECSKTGTCNCGGSCTCKNCSCTTCNK SCCPCCPSGCPKCASGCVCKGKTCDTTCCQ (1)
----------S-------K-S--A--S-K-A---D------S---------------S--- (2)
----------S-------K-S--A--S-K- --------D-S---------------S--- (3)
---------------AT-K-T-------K- ---F------S--------N-NS-GSS--- (4)
------A-----------S-TK---KS-K- ---D------S---------------S--- (5)
```

(1)Winter Flounder MT, adapted from Chan *et al.*, 1989.

(2) Rainbow Trout MT-A, adapted from Bonham *et al.*, 1987.

(3) Rainbow Trout MT-B, adapted from Bonham *et al.*, 1987.

(4) Stone-Loach MT, adapted from Kille *et al.*, 1991.

(5) Tilapia MT, adapted from Chan, 1994.

Fig.1.4 Alignment of fish MT Amino Acid Sequences. Metallothioneins are highly conserved in Teleosts and the 20 Cysteine Residues Remain Unchanged.

Chapter 2 MT mRNA Induction of Tilapias after Intraperitoneal Injection of Metal

2.1 Introduction

Ever since the discovery that metallothionein (MT) was identified as a cadmium binding protein in equine kidney cortex (Margoshes *et al.*,1957), it has also been demonstrated to bind with other essential trace elements such as zinc and copper (Kagi *et al.*,1960; Pulido *et al.*,1966). Therefore, it is believed that MT played an important role in homeostasis of essential metal ions such as copper and zinc (Bremner,1987), in addition to its role of detoxification of non-essential metals such as cadmium and mercury (Coogan *et al.*,1994; Cosson,1994).

Previous studies showed that untreated common carp (Kito *et al.*,1986) and some of the feral carp species (Kito *et al.*,1982) had basal hepatic and renal MT expression. Furthermore, it was shown that exposure to cadmium induced the synthesis of MT to reduce their toxicity in several fish species including killifish (Koizumi *et al.*,1992), perch (Olsson *et al.*,1986) and white sucker (Klaverkamp *et al.*,1987). Although MT levels increased in response to metal exposure, changes in MT levels might not be in the same extents in different tissues. For example, George *et al.* (1996) reported that MT levels decreased in the order: liver > kidneys > gills in the turbot with cadmium intraperitoneal injection. Comparing with MT levels, MT mRNA levels in cadmium-injected turbot were induced to similar levels in the three tissues but could maintain elevated levels continuously at higher dose of cadmium administered (>200μg/kg) (George *et al.*,1996). In addition, it gave more rapid and significant MT mRNA induction levels after single 75μg/kg cadmium administered (George *et al.*,1996). Therefore, MT mRNA seems to be good parameter to indicate the response of fish exposed to metals.

Northern blot analysis, which involves a specific MT cDNA probe, is one of the methods to estimate MT mRNA levels. Since tilapias MT cDNA probe was cloned in our laboratory, MT mRNA levels in tilapias with metal administrations were measured using northern blot analysis in the present study.

2.1.1 Specific Aims of this Chapter

Cadmium, copper and zinc were tested separately in tilapias and only zinc injection was tested in carp for comparison. Saline-injected species were used as controls. In addition, tilapias without any chemical injection indicated the endogenous levels of MT mRNA in normal conditions.

Both tilapias MT cDNA (Chan,1994) and carp MT cDNA probes (Chan,1996) with about 400 bp are very specific for tilapias and common carp respectively were employed to study the endogenous and metal induced MT mRNA levels in different tissues of tilapias and common carp using northern blot analysis.

The aims of the experiment are listed as follows:

1. To determine the basal level of MT mRNA in different tissues of tilapias

2. To show the level of MT mRNA induction in different tissues of tilapias injected with metals.

3. To compare MT gene expression of tilapias and common carp after metal injections.

2.2 Materials and Methods

2.2.1 Regents

2.2.1.1 Purification of Total RNA

Diethyl pyrocarbonate (DEPC) treated water:

add 1 ml DEPC to 1 L double distillated water (dd H_2O)
stirred overnight and autoclaved

Extraction Buffer for Total RNA Preparation (Chomczynski *et al.*, 1987):

25 g of guanidium thiocyanate
1.76 ml of 0.75M sodium citrate, pH 7.0
2.64 ml of 10% N-lauroylsacrosine sodium salt
dissolved in 29 ml of autoclaved DEPC-treated ddH_2O at 65°C
360 µl of β-mercaptoethanol added
adjusted to 50 ml autoclaved DEPC-treated ddH_2O

Phenol, Tris-saturated:

add 1 volume autoclaved 1M Tris-Cl, pH 8.0
stirred for 3 hours and the aqueous phase were removed.
add 1 volume of autoclaved 0.1M Tris-Cl, pH 8.0
stirred for 3 more hours.

2.2.1.2 Denaturing Gel and Vacuum Blotting of RNA (Northern Blotting)

3-(N-morpholino)-propanesulfonic acid (MOPS) Buffer (10X) for agarose gel electrophoresis:

41.9 g MOPS
6.8 g sodium acetate
20 ml of 10mM ethylenediaminetetraacetic acid (EDTA), pH 8.0
made up to 1 L with ddH_2O and pH adjusted to 7.0
treated with 1 ml DEPC, stirred overnight and autoclaved

Sample buffer:

2 µl of 10X MOPS
3 µl 37% formaldehyde (MERCK)
10 µl Stop Solution (Pharmacia)

Standard Saline Citrate (SSC) (20X) for transfer buffer:

175.3g sodium chloride (NaCl)
88.2g Sodium Citrate
made up of 1L with ddH_2O, pH adjusted to 7.0 and autoclaved

1 % agarose gel for RNA:

1 g agarose
10 ml 10X MOPS Buffer
85 ml DEPC-treated ddH_2O and then melt the agarose in an oven
5 ml 37% of formaldehyde
Pour the mixture into the gel tray after cooling and wait until the gel polymerized

2.2.1.3 Hybridization

Rapid Hybridization Buffer (Life Technologies)

2.2.2 Methods

2.2.2.1 Purification of Total RNA

Total RNA extraction from different tissues of tilapias was prepared either according to (Chomczynski *et al.*,1987) or using TRIzol™ Reagent (Life Technologies). The amount of tissues used was 80 to 150 mg, except for muscle (1 g tissue was used instead).

Tissues of 100mg (small scale preparation) were homogenized with 1 ml TRIzol™ Reagent which contains phenol in the extraction buffer as described above. Total RNAs were further extracted with 0.1 volume of 1-bromo-3-chloropropane (BCP), and precipitated by adding 1 volume of isopropanol. The RNA precipitated was then washed by at least 1 ml 70% ethanol. The precipitated RNA samples were resuspended in DEPC-treated ddH_2O.

In this method, the total RNA samples were dissolved in 50 µl DEPC-treated distilled water (for total RNAs from livers, 200 µl DEPC-treated distilled water was used) and then their optical densities were meausured at 260 nm and 280 nm wavelength ($O.D._{260}$ and $O.D._{280}$ respectively) using fluorescence spectroscopy. The qualities of the total RNA samples were

determined by the ratio of $O.D._{260}$ to $O.D._{280}$. The concentrations were estimated by assuming 1 $O.D._{260}$ unit equals to 40 μg/μl for single stranded nucleic acid. The total RNA samples were ready for northern blot analysis.

2.2.2.2 Vacuum Blotting of Total RNA (Northern Blotting)

Total RNAs (10 to 20 μg in 5 to 10 μl) from different tissues were mixed with 10 μl formaldehyde, 3 μl formaldehyde gel-loading buffer, 2 μl ethidium bromide (1μg/μl) and 2 μl 10X MOPS and then resolved in formaldehyde agarose gel. Formadehyde was washed away by soaking with 2X SSC (DEPC-treated) after electrophoresis at 100V until the dye migrated two third of the gel. RNA was fixed, after transferred to nylon membrane in 10X SSC via vacuum blotting with ultraviolet (UV) crosslinking using UV Crosslinker (Fotodyne).

2.2.2.3 Radioactive Labeling of Nucleic Acid Probes

DNA fragments separated on gels were purified using Sephaglas™ BandPrep Kit (Pharmacia). Purified DNA fragments (about 400 ng) were labeled with α-^{32}P-dCTP (PB10205, Amersham) using Nick Translation (N5000, Amersham). The unincorporated α-^{32}P-dCTP was removed by Sephadex G-50 spin-column chromatography (Maninatis, *et al.*,1982). Over 60% of radioisotopes were incorporated and the probe usually estimated having a specific activity of 10^8 cpm/μg DNA after nick translation.

2.2.2.4 Hybridization

Nylon membrane was hybridized with denatured ^{32}P-labeled probe for 2 hours. After hybridization, the membrane was washed with 2X SSC/ 0.1% SDS at room temperature once, and finally at 65°C twice. Autoradiography was carried out with either Kodak XO-mat-AR film or Kodak BioMax film.

2.2.2.5 Densitometric Analysis

For quantification purposes, the relative intensities of the signals from the northern blot (MT or Actin mRNA) or the positive film of agarose gel (18S rRNA) were quantified using a PC version of ImageQuaNT program (Molecular Molecular Dynamics). The autoradiogram or negative films of gel-picture were scanned using a Personal Imager (Molecular Dynamics). The 18S signals from the negative film were used to normalize the northern blot signals. Fold induction of MT mRNA in the tissues of tilapias was defined by dividing MT mRNA levels after metal injection with MT mRNA levels after saline injection.

2.2.2.6 Calculation of MT mRNA levels and Analysis of Results

Firstly, the hybridization signals of MT mRNA in X-ray films were normalized by 18S rRNA signals from the negative film of the agarose gels. The normalized values of the treated and untreated tilapias and carp were analyzed statistically (ANOVA and correlation analysis) with procedures in the Microsoft Excel package. The 5% confident level of significance was chosen for the statistical analysis.

2.2.3 Endogenous MT mRNA Expression of Tilapias

Tilapias and common carp were obtained from the Agriculture and Fisheries Department, Hong Kong Government. Their weights ranged from 90 to 100 g and 70 to 100 g, respectively. After kept in the aquaria for 2 weeks to acclimate the laboratory conditions, different tissues were removed and total RNA samples were prepared according to the method described by Chomcznski and Sacchi (Chomczynski *et al.*,1987). Total RNA (10 μg) from different tissues were used for northern blot analysis. The blots were

hybridized with α-^{32}-P-dCTP labeled full length tilapias MT cDNA (Chan,1994) and carp MT cDNA probes (Chan,1996)using Rapid Hybridization Buffer (Amersham) and the washing conditions were the same as that described in Section 2.2.2.4. Autoradiographs were developed in Kodak XO-mat film with double intensifying screens at -70°C for 96 hours.

2.2.4 Induction of MT mRNA Tilapias Injected with Metals

Similar size and weight of tilapias (91-106 g) and carp (456-704 g) were chosen and then kept in the aquaria for 2 weeks to acclimate the environment. They were not fed 3 days before the experiment started. Each fish species was divided into 2 groups (3 tilapias or carp each group): the saline-injected group and metal-injected group such as cadmium (II) chloride, copper (II) chloride and zinc (II) sulphate in the following dosages:

Time	Cadmium (mg/kg)	Copper (mg/kg)	Zinc (mg/kg)
Day 1	0.6	0.6	0.6
Day 2	0.8	0.8	0.8
Day 3	1.0	1.0	1.0
Day 4	2.0	2.0	3.0

At Day 5, they were sacrificed and the tissues (brain, gill, intestine, kidney, liver and muscle) were freshly removed and immersed in liquid nitrogen. Tissues were kept at -70°C for northern blot analysis. Simultaneously, the saline-injected group as the control was treated with 0.9% saline injections for 4 consecutive days.

2.3 Results

2.3.1 Endogenous Levels of MT mRNA in Tilapias in normal conditions

In Fig. 2.1, both tilapias and carp showed higher endogenous MT mRNA levels in their intestine and brain under normal conditions. Also, tilapias showed lower endogenous levels of MT mRNA than carp except in the gills.

2.3.2 Induction of MT mRNA Levels in Tilapias Injected with Metals

2.3.2.1 Copper Injections

The results of northern blot analysis are shown in Fig.2.2. The results indicated that all the tissues had higher MT mRNA induction levels except gills and kidney, which showed no observable difference after copper injection. The highest fold induction of MT mRNA in tilapias injected with copper was liver which was 85-fold of MT mRNA induction level more than that from saline-injected tilapias. Muscle, brain and intestine were 25, 3.1 and 1.5 fold induction respectively (Table 2.1).

2.3.2.2 Zinc Injections

The results of northern blot analysis are shown in Fig. 2.2. According to the results, all the tissues showed higher MT mRNA levels in zinc-injected than saline-injected tilapias especially in liver, kidney and intestine which were high significant different than those from saline-injected species ($p<0.05$ in gills and intestine, $p<0.01$ in liver). It indicated kidney was the highest MT mRNA levels in zinc-treated tilapias. The fold inductions of MT mRNA level were 10 and 5.7 in gills and liver respectively which were higher than brain, intestine and muscle (Table 2.1).

2.3.2.3 Cadmium Injections

The results of northern blot analysis are shown in Fig. 2.4. From the results, no significant different of MT mRNA levels between saline and cadmium injected tilapias were obtained except in liver ($p<0.05$) which was 3.1 fold higher in cadmium injected tilapias than saline-injected tilapias (Table 2.1). In contrast, the means of MT mRNA levels in kidney and intestine of saline-injected tilapias were higher than cadmium-injected tilapias.

2.3.3 Induction of MT mRNA levels in Carp with Zinc Injections

The results of northern blot are shown in Fig. 2.5. Except brain and gills, all other tissues showed higher MT mRNA levels in zinc-injected carp than saline-injected carp. In addition, kidney was the tissue, which showed the highest induction of MT mRNA levels. Intestine and liver were the second and third respectively. Muscle was higher MT mRNA level than gills and brain in zinc-injected. However, brain and gills showed declined MT mRNA levels in zinc-injected than saline-injected carp (Table 2.1).

2.4 Discussions

2.4.1 MT mRNA Expression of Tilapias and Carp Injected with Metals

By using homologous MT cDNA probes from tilapias and carp, significant basal MT mRNA levels were determined in tilapias and carp. Comparing MT mRNA levels in the tissues of tilapias with carp, they had similar endogenous MT mRNA levels. The presence of endogenous MT mRNA levels indicated that the protein might have important basic physiological functions. The variations in endogenous MT mRNA levels between different organs might

be due to differences in the role of each tissue in metal-ion metabolism. For instance, the highest endogenous level of MT mRNA in tilapias and carp were intestine which was believed to relate MT level with absorption of metal ions in rat (Richards *et al.*,1977). However, Shears *et al.* (1984) examined the involvement of MT in gastrointestinal zinc uptake but found no enhancement or depression of zinc uptake was associated with MT concentration in the intestinal cytosol of winter flounder.

The present investigation indicated different tissues had various levels of MT gene expression after metal administration as demonstrated previously *in vivo* (Chan *et al.*,1989; Zafarullah *et al.*,1989). Liver was the highest MT mRNA induction level in tilapias after metal injection. In a similar study on rainbow trout intraperitoneally injected with cadmium, MT mRNA induction was increased in liver, kidney and gills; especially in liver, hepatic MT mRNA showed the highest induction level (Norey *et al.*,1990). These results indicated that liver is an essential organ for metal detoxification.

The MT mRNA level of kidney in saline-injected tilapias was found to be high. This response might be related to stress. Although MT mRNA level was higher in the tissues of metal-injected animals, the MT synthesis has been reported not to parallel changes in MT mRNA level. Vasconcelos *et al.* (1996) reported that the regulation of MT isoform expression by copper and cadmium involved differential translational control in rat. But in the present study, no MT levels in different tissues were studied. According to Fleet *et al.* (1988), tissue-specific MT accumulation depended on the route of zinc administration in chicken. More MT accumulation following intraperitoneal injection was obtained while the pancreas accumulates more MT after intravenous or oral administration.

In the present study, the MT mRNA level in kidney was greater than liver in both carp and tilapias injected with zinc. Similar results were obtained in

cadmium-injected carp (Chan,1996). Kidney seemed to be major sink of cadmium for fishes exposed to cadmium in water in form of Zn,Cu,Cd-binding protein, presumably MT, but not in the gills and liver (Petering *et al.*,1990).

Relatively lower as compared to fish MT mRNA level was obtained in the brain and 3 isoforms of MT (MT-I, MT-II and MT-III) were found to be expressed constitutively in the brain of mouse (Klaassen *et al.*,1996). Kramer *et al.* (1996) reported that cadmium and zinc stimulated the increases in both MT-I and MT-II mRNA levels, whereas MT-III mRNA production was relatively unresponsive to metal inductions. MT-III was not inducible by metals but it played some roles in preventing neuronal sprouting and development of neurofibrillary tangles (Palmiter *et al.*,1992). MT-III however, could sequester zinc from the synapse, maintaining homeostatic control over neurotransmitter levels with the synaptic cleft (Aschner,1996). No MT-III and IV are reported in fish brain so far.

From the results, a relatively low MT mRNA induction level was found *in vivo* in the gills of tilapias and carp injected with metals. At low dose (100 μg/kg) of cadmium injections, lower MT mRNA level was found in the gills than liver and kidney of turbot (George *et al.*,1996).

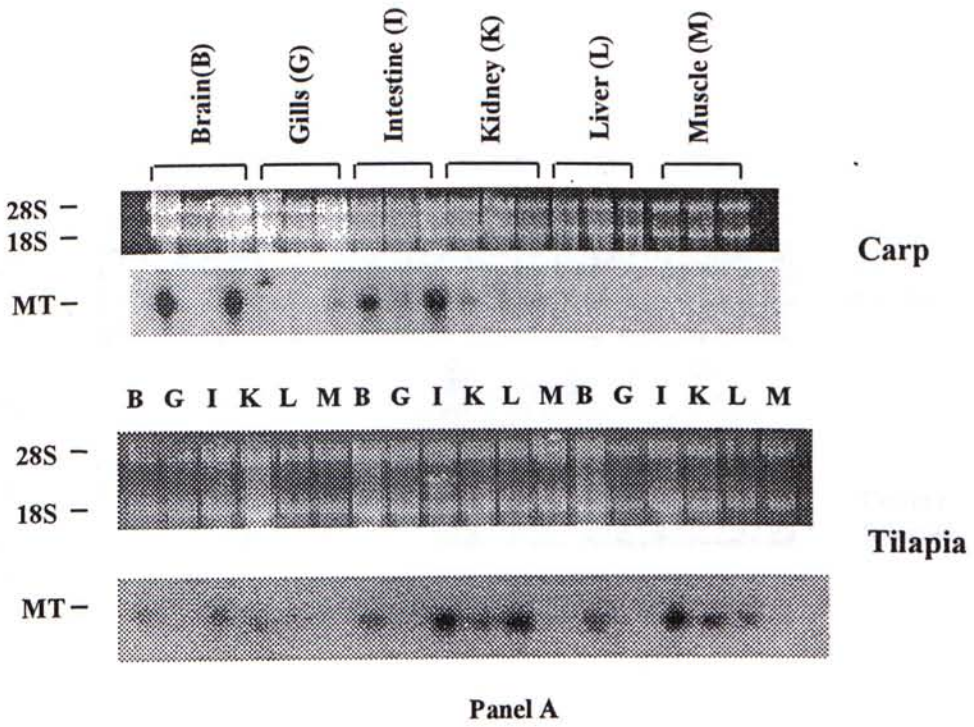
Teleost MTs can be induced by a variety of heavy metal ions in different extents. In flounder, the induction of MT mRNA by cadmium was higher than copper (Chan *et al.*,1987). In the present study, MT mRNA levels induced by zinc as well as cadmium was greater than copper in liver, kidney and intestine of tilapias.

2.5 Conclusion

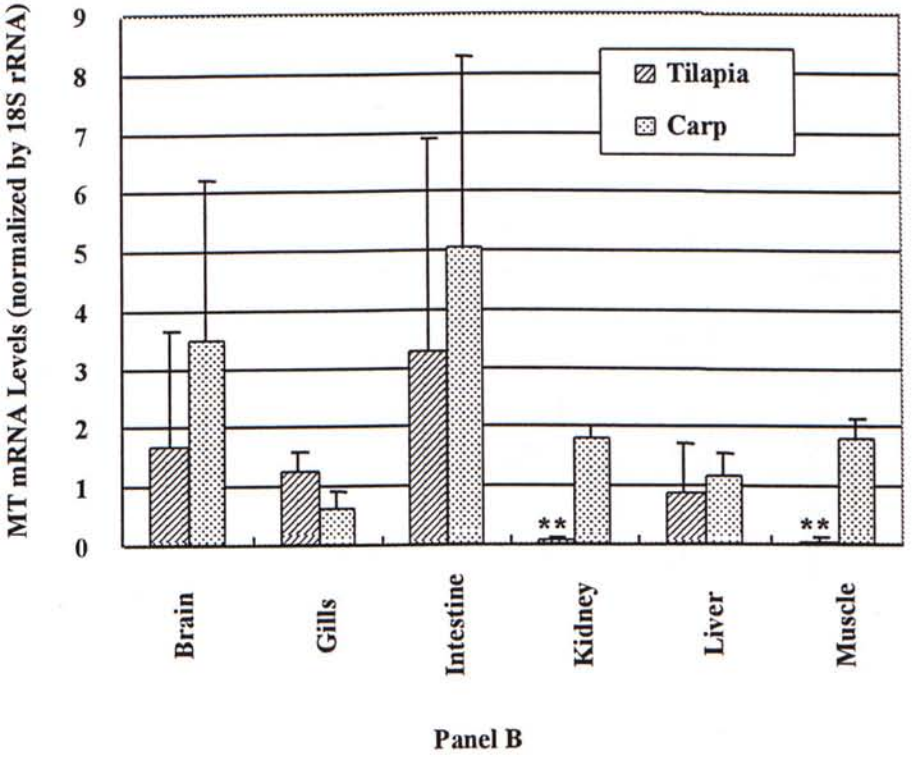
In conclusion, both tilapias and carp had similar endogenous level of MT mRNA in different tissues. Intestine showed the highest endogenous level of MT mRNA and liver with a lesser extent of MT mRNA levels. All the tissues of tilapias injected with metals induced higher level of MT mRNA than saline-injected tilapias except in kidney of saline-treated tilapias. In addition, liver showed the highest fold induction of MT mRNA, except in the zinc-injected tilapias, which showed higher induction level of MT mRNA in the kidney. Also in zinc-injected carp, higher level of MT mRNA induction was found in the kidney than in the other tissues.

Tissues	Tilapia			Carp
	Copper	Zinc	Cadmium	Zinc
Brain	3.1	3.6	0.7	0.2
Gills	1.1	10	1.7	0.54
Intestine	1.5	2.4*	0.7	9*
Kidney	1	3*	0.2	30*
Liver	85	5.7**	3.1*	2.1
Muscle	25	2.2	1.7	4

Table 2.1 Fold induction of MT mRNA in the tissues of metal-injected tilapia compared with saline-injected tilapia. This table showed the fold inductions of MT mRNA are obtained by dividing MT mRNA levels in metal injected tilapia with MT mRNA levels in saline injected tilapia. "**" and "*" represents $p < 0.01$ and $p < 0.05$ respectively.

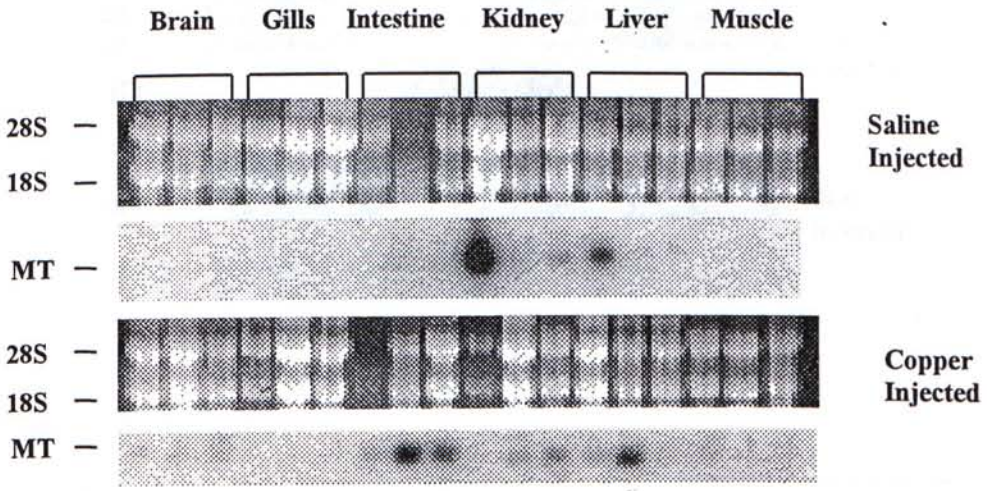


Panel A

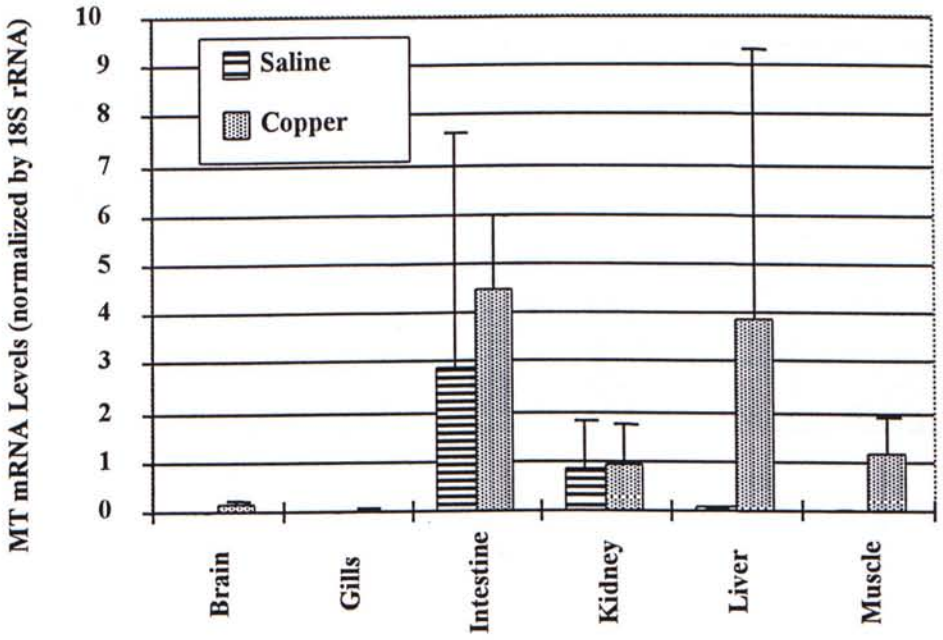


Panel B

Fig. 2.1 Northern Blot Analysis showing Endogenous Levels of MT mRNA in tilapia and Carp in Normal Conditions. Panel A shows the result of the northern blot of three tilapia and carp. **Panel B** The optical densities of the hybridization signals of MT mRNA levels in different tissues were normalized with 18S rRNA. The normalized data are grahphically presented as mean± SD (n=3).("**" represents $p<0.01$; compared with MT mRNA levels in carp)

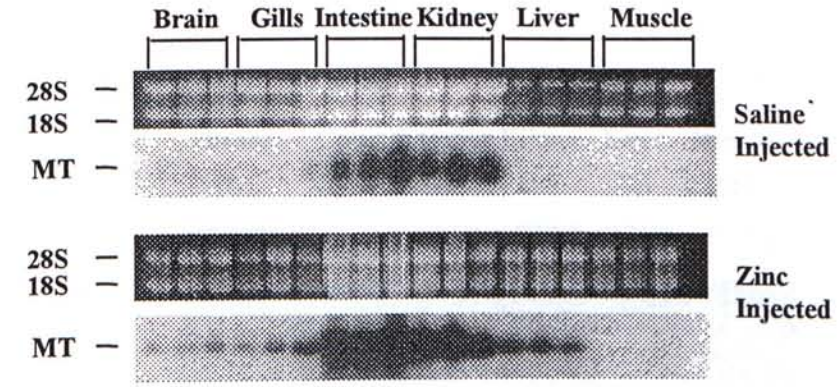


Panel A

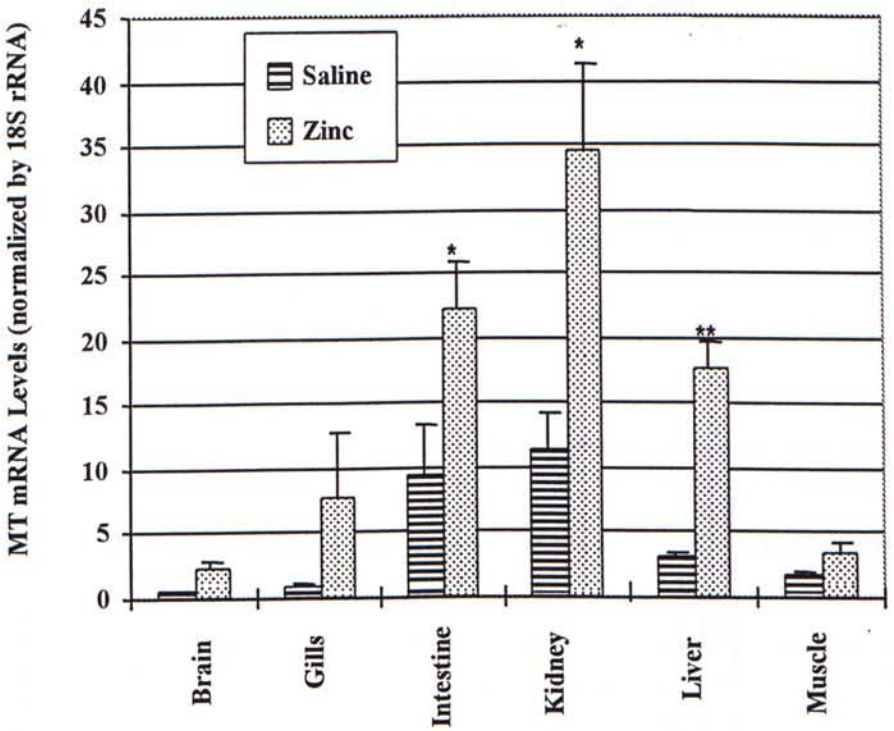


Panel B

Fig. 2.2 Northern Blot Analysis Showing the Induction of MT mRNA Levels in Tilapia after Copper Injections. Panal A shows the result of the Northern blot of three tilapia with saline-injections and copper injections. **Panel B** The optical densities of the hybridization signals of MT in different tissues were normalized with 18S rRNA. The normalized data are graphically presented as mean ± SD (n=3).



Panel A



Panel B

Fig. 2.3 Northern Blot Analysis Showing the Induction of MT mRNA Levels in tilapia after Zinc Injections. Panal A shows the result of the Northern blot of three tilapia with saline-injections and zinc injections. **Panel B** The optical densities of the hybridization signals of MT mRNA in different tissues were normalized with 18S rRNA. The normalized data are graphically presented as mean± SD (n=3)("*" and "**" represents $p<0.05$ and $p< 0.01$ respectively; compared with MT mRNA levels in saline-treated tilapia).

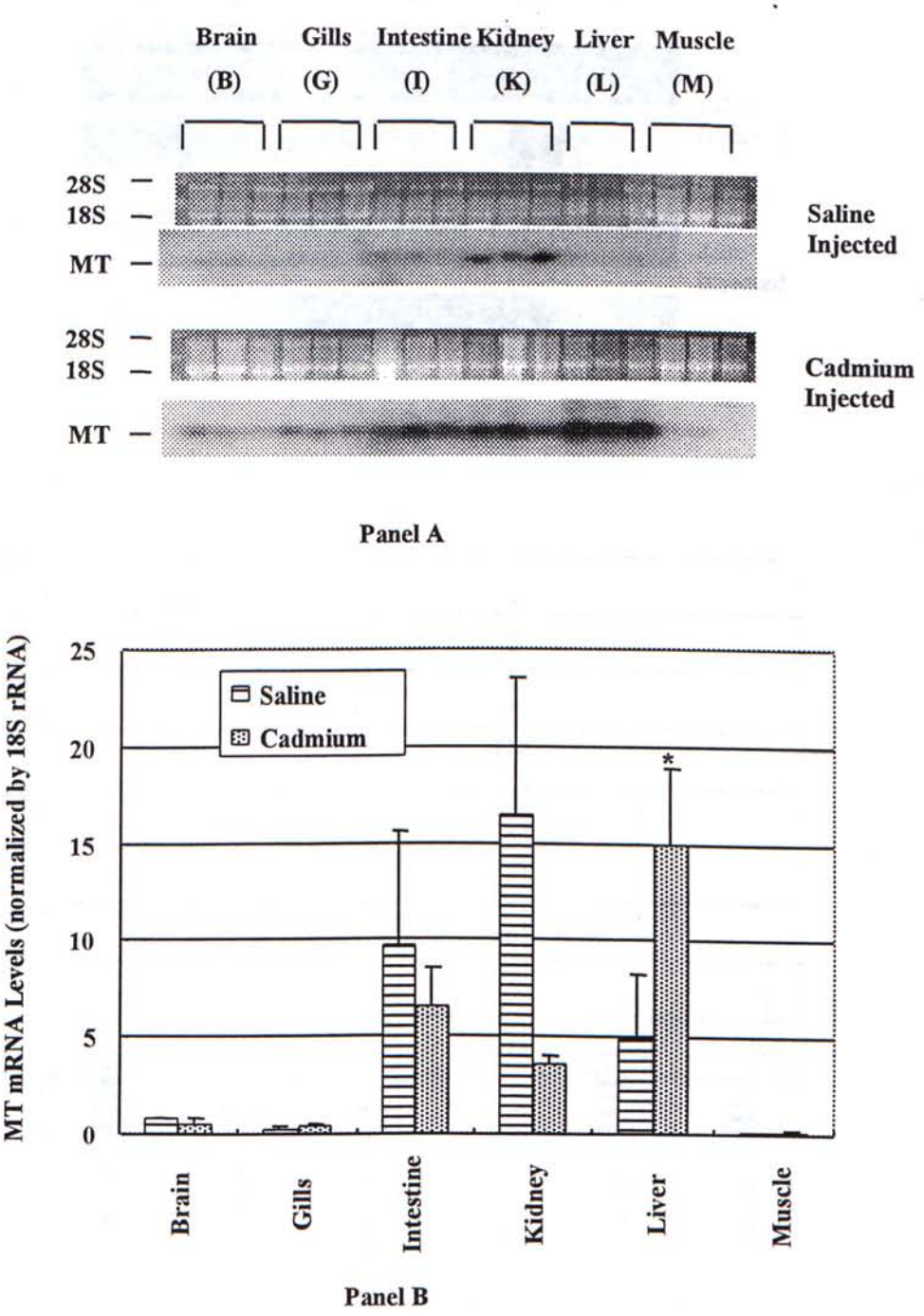
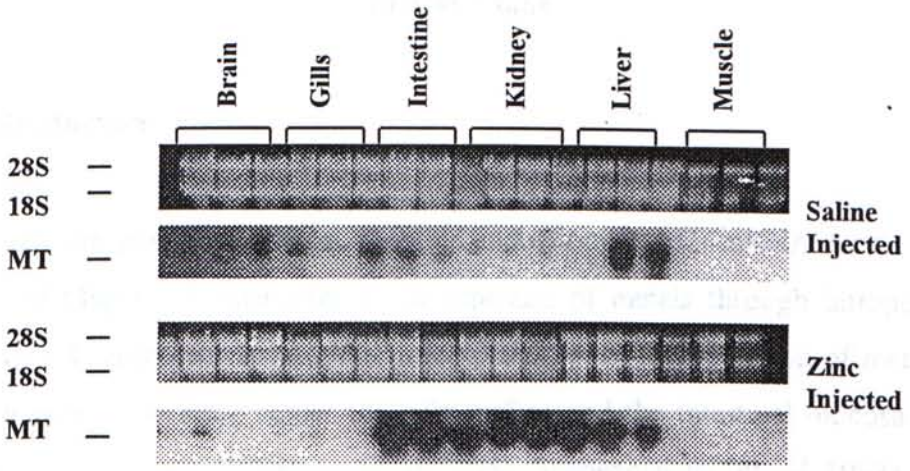
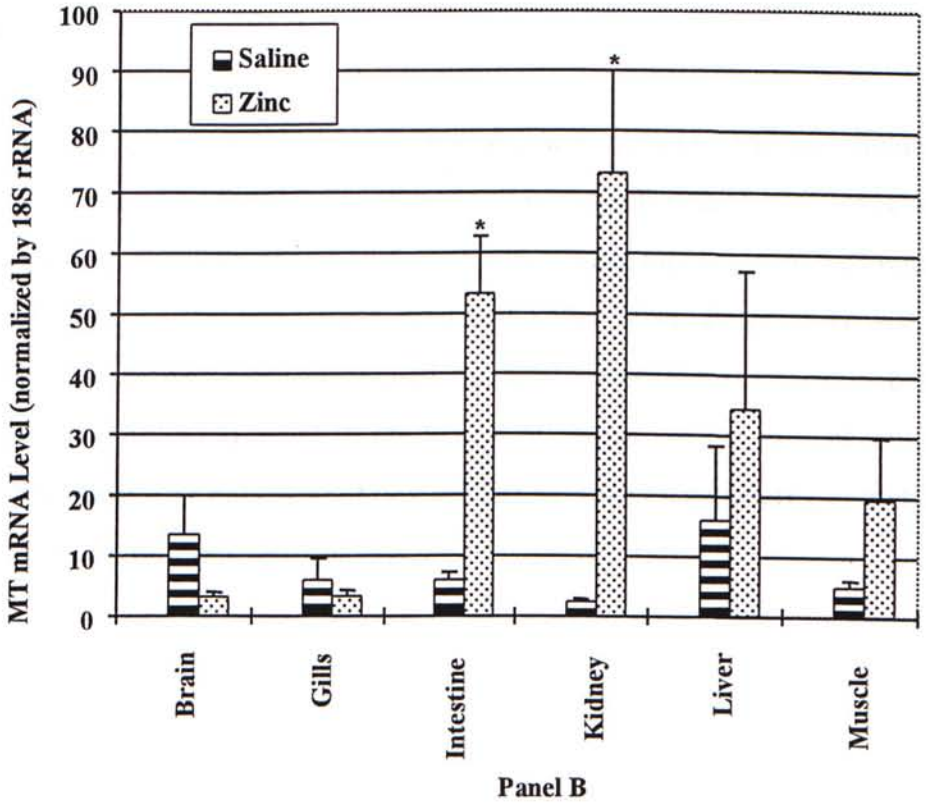


Fig. 2.4 Northern Blot Analysis Showing the Induction of MT mRNA Levels in tilapia after Cadmium Injections. Panal A shows the result of the Northern blot of three tilapia with saline-injections and cadmium injections. **Panel B** The optical densities of the hybridization signals of MT in different tissues were normalized with 18S rRNA. The normalized data are graphically presented as mean ± SD (n=3)("*" represents $p<0.05$; compared with MT mRNA levels in saline-treated tilapia).



Panel A



Panel B

Fig. 2.5 Northern Blot Analysis Showing the Induction of MT mRNA Levels in carp after Zinc Injections. Panal A shows the result of the Northern blot of three carp with saline-injections and zinc injections. **Panel B** The optical densities of the hybridization signals of MT in different tissues were normalized with 18S rRNA. The normalized data are graphically presented as mean ± SD (n=3)("*" represents $p<0.05$; compared with MT mRNA levels in saline-treated carp).

Chapter 3 Induction Levels of MT mRNA in Tilapia after Aqueous Exposures to Metal Ions

3.1 Introduction

In the previous chapter, the fold induction of MT mRNA levels in the tissues of tilapia and carp after acute exposure of metals through intraperitonal route was determined. In fact, the major pathways of incorporation of metal ions for fish were absorption across the gills surface and the intestinal mucosa in the aquatic environment (Williams *et al.*,1978; Schoenmakers *et al.*,1992). For example, the gills of fish were the principal sites for exchange of these soluble waterborne metals (transferred into the circulatory system by binding with metal binding proteins including branchial MT) after aqueous exposure of sublethal doses of cadmium (Kito *et al.*,1982).

There were several studies of the sublethal toxicity test and accumulation of metals in tilapia. The distribution of mercury in the soft tissues of *Tilapia aureus* and interactions between copper and cadmium modify metal organ distribution in mature *Tilapia mossambicus* were examined (Allen,1994; Pelgrom *et al.*,1995). However, few studies were performed on determining the relationship between metal and MT induction levels in the tissues of tilapia. In the present study, the metal dose-dependent induction of MT mRNA in aqueous exposures to metal ions was assessed by northern blot analysis.

3.1.1 Specific Aims of this Chapter

The aims of this experiment are:

1. To determine the relationship between fold induction of MT mRNA in the tissues of tilapia and carp exposed at sub-lethal metal concentrations.

2. To compare the fold induction of MT mRNA levels in the tissues of tilapia and carp.

3.2 Materials and Methods

3.2.1 96 hours LC-50 values for zinc and copper

Twenty juvenile tilapia with similar length and weight were distributed randomly in several aquaria, which were presoaked with 0.5% nitric acid to remove metal residues. They were exposed to serial diluted concentrations of metals as follows:

Zinc Conc. (mg/L)	0	10	15	20	25			
Copper Conc. (mg/L)	0	0.05	0.1	0.25	0.5	1.0	2.5	5.0

The aquaria were replenished everyday with new tap water which were stayed overnight. The experiment was conducted for 96 hours. Everyday the temperature and pH values were monitored. Mortality of the tilapia were also recorded and their fork lengths and weights were measured every 24 hours. The pH range during the experiments was 6-8. The temperature range was 19-24°C. The total volume of water added into those tanks was adjusted to 10 liters. The LC-50 values were calculated using a graph of survival rate against metal concentration in log scale.

3.2.2 Induction of MT mRNA in Juvenile Tilapia under Metal Aqueous Exposures

Tilapia and carp (juvenile or fingerling) weighing between 10 and 15 g, 9 and 12 g respectively, were obtained from Agriculture and Fisheries Department, Hong Kong Government. They were kept in the aquaria, which were treated with 0.1% HCl overnight and then wash to remove any the metal residues contaminated. Later, the aquaria were filled with 10 litres of tap water. The fish were allowed to acclimate for 2 days. Then, they were exposed to 3 different doses of metals for 3 weeks (10, 50 and 100μg/L for copper, cadmium and zinc but three additional doses (0.4, 1 and 2 mg/L) for zinc). Half of the water in the aquaria was changed everyday in the morning with tap water, which was kept overnight. Water samples and tissues of juvenile tilapia were collected and three fish were removed at Week 0, 1, 2 and 3 respectively and kept at -70°C until use.

The total RNA samples from the tissues of tilapia and carp exposed to metals were extracted using TRIzol reagent (Section 2.2.2.1). Ten micrograms of the total RNA from different tissues were resolved in a 1% denaturing agarose gel in 1X MOPS buffer and then transferred on to Hybond N (Amersham) nylon membrane (Section 2.2.2.2). The membrane was hybridized with a α-32P-dCTP labeled full-length MT cDNA in Rapid Hybridization Buffer (Amersham) (Sections 2.2.2.3 and 2.2.2.4). The washing conditions were described as before (Section 2.2.2.4) except with an additional wash of 0.5X SSC/1% SDS at 65°C for half an hour. Autoradiograph was developed in Kodak XO-mat film for 96 hours with double intensifying screens at -70°C.

3.2.3 Calculation of Fold Induction of MT mRNA and Analysis of Results

The hybridization signals of MT mRNA in X-ray films were normalized by actin mRNA and presented as fold induction over controls at the same time of exposure. Fold induction of MT mRNA levels was defined by dividing the normalized values of the tilapia and carp exposed to metals with those of control tilapia and carp, which were not exposed to metals. Statistical analysis (ANOVA and correlation analysis) was employed with procedures in the Microsoft Excel package. The 5% confident significance level was chosen for the statistical analysis.

3.2.4 Metal Analysis

Fifty ml of water samples from the aquaria at Week 1, Week 2 and Week 3 were collected and acidified with the addition of a few drops of nitric acid to 0.5% v/v. The acidified samples were then stored at 4°C before metal measurement by atomic absorption spectrophotometer (Flame SpectrAA-10; Zeeman SpectrAA-800, Varian).

3.3 Results

3.3.1 LC50 values of Metals for Juvenile Tilapia

The LC50 values were determined by the plot of percentage mortality against the dosage in log scale. The dosage corresponds to 50% mortality was chosen at the LC50 values. The copper and zinc toxicity curves for tilapia were attached at the appendix and the LC50 values were showed below:

Metals	24 hour LC50	96 hour LC50
Copper	2.82 mg/L	1.52 mg/L
Zinc	24.3 mg/L	16.5 mg/L

Table 3.1 24 hour and 96 hour LC50 values of copper and zinc in tilapia

Comparing with 24 hour metal toxicity curve, 96 hour metal toxicity curve of tilapia showed similar patterns but shifted a little bit left from 24 hour metal toxicity curves (appendix 1 and 2). Consequently, the 96 hour LC50 values for copper and zinc were lower than those for 24 hour exposure. In the table 3.1, the 24 hour and 96 hour LC50 values were 2.82 and 1.52 mg/L for copper, and 24.3 and 16.5 mg/L for zinc.

3.3.2 Induction of MT mRNA in Juvenile Tilapia under Metal Aqueous Exposures

3.3.2.1 Aqueous Exposure to Copper

The results of fold induction of tilapia MT mRNA in the liver and gills were shown in Fig 3.1 and 3.2 respectively. In Fig. 3.1, the fold induction of MT mRNA in the liver showed no significant difference. No significant correlation between fold induction of MTmRNA and time course at lower doses was obtained. However, exposure to 100 μg/L of copper, a positive correlation was found (r=0.726) (Table 3.5).

From Fig. 3.2, elevated levels of fold induction of MT mRNA in gills at 10 and 50 μg/L (r=0.876, 0.842 respectively) (Table 3.5) were obtained after 3 weeks exposure. However, at 100 μg/L, negative correlation in the gills was obtained

(r=-0.862) (Table 3.5). As well as in the liver, no significant fold induction of MT mRNA in the gills were determined.

3.3.2.2 Aqueous Exposure to Zinc

The results of fold induction of MT mRNA in the liver and gills of tilapia were shown in Fig. 3.3 and 3.4. Tilapia exposed to zinc showed that there was a direct correlation of fold induction of MT mRNA in the liver and time course at 10 and 50 μg/L (r=0.816, 0.978 respectively) (Table 3.5). There was a peak of fold induction of MT mRNA in week 1, but negative relationship with time course at 100 μg/L of zinc exposure (r=-0.754) (Table 3.5) was found.

In Fig. 3.4, there were little correlations between fold induction of MT mRNA over time at 50 and 100 μg/L (r=0.875, 0.627 respectively) (Table 3.5). However, an unexpected higher fold induction in week 2 at 10 μg/L of zinc exposure was detected. At Week 3, the fold induction of MT mRNA was significant correlated with zinc concentrations (r=0.950) (Table 3.5).

The results for tilapia exposed to higher doses of waterborne zinc (0.4, 1 and 2 mg/L) were shown in Fig 3.5. From Fig. 3.5, it showed direct correlation between fold induction of MT mRNA and exposure concentration (0.4, 1 and 2 mg/L) at Week 1 and Week 2 in the liver of tilapia (r=0.942 and 0.974 respectively) (Table 3.5) and Week 2 in the gills (r=0.845) (Table 3.5). However, no significant correlation between fold induction of MT mRNA in the gills and exposure concentration was obtained at Week 1 (r=0.079) (Table 3.5).

3.3.2.3 Aqueous Exposure to Cadmium

The results of fold induction of tilapia MT mRNA were shown in Fig. 3.6. It was found that there were little correlation between fold induction of MT mRNA in liver and time course at 10 and 50 μg/L (r=0.540, 0.813 respectively) (Table 3.5). At 100 μg/L of cadmium exposure, however, a declined trend of fold induction with time was found (r=-0.559) (Table 3.5).

The fold induction of MTmRNA in the gills of tilapia exposed to 10, 50 and 100 μg/L of cadmium was shown in Fig. 3.7. Ten μg/L of cadmium exposure gave significant correlation between fold induction of MT mRNA in the gills and time course (r=0.998) (Table 3.5) but this relationship was lower at higher doses (r=0.717, 0.831 for 50 and 100 μg/L respectively) (Table 3.5). The fold induction of MT mRNA at 100 μg/L was relatively lower than 10 μg/L and 50 μg/L.

3.3.3 Induction of MT mRNA in Juvenile Carp after Aqueous Exposures to Metal

3.3.3.1 Aqueous Exposure to Cadmium

The results of fold induction of MT mRNA in the liver and gills of tilapia were showed in Fig. 3.8 and 3.9. Comparing the fold induction in the liver with different dosages in Fig.3.8, no significant correlation was determined.

From the result of Fig. 3.9, there was the highest fold induction of MT mRNA in Week 3 at 10 μg/L cadmium exposure. The fold induction of MT mRNA correlated directly over time (r=0.874 for 10 μg/L) (Table 3.5) but at higher doses, negative correlations were obtained (r=-0.976, -0.172 for 50 and 100 μg/L respectively) (Table 3.5).

3.3.4 Metal Concentrations of Water Samples from the Aquaria in the Metal Exposure Test of Tilapia and Carp

The water samples from the aquaria in sublethal exposure test were measured. From the table 3.2, waterborne zinc in control was higher in tilapia than carp.

Species	Metals		Control	10μg/L	50μg/L	100μg/L
Carp	Cadmium	Mean	1.17	10.32	49.98	87.56
	(μg/L)	s.d.	0.81	2.631	11.18	22.76
Tilapia	Cadmium	Mean	1.95	10.55	38.7	57.06
	(μg/L)	s.d.	1.00	0.88	2.61	7.60
Tilapia	Copper	Mean	0.91	10.88	41.31	93.69
	(μg/L)	s.d.	0.45	1.78	15.68	6.73
Tilapia	Zinc	Mean	157.47	142.23	162.6	253.48
	(μg/L)	s.d.	128.001	46.034	16.63	39.25

Table 3.2 Concentrations (mean and standard deviation, s.d.) of waterborne metals in aqueous exposure tests

3.4 Discussion

3.4.1 LC50 values of Metals for Tilapia

From the result in Table 3.1, the 96 hour LC50 value of zinc for tilapia was quite similar to the other species although experimental temperature in the present study was higher. However, the toxicity of copper among fish species were variable with 96 hour LC50 values ranging from 0.05 mg/L in carp to 0.46-0.49 mg/L in fathead minnow. Tilapia was the highest 96-h LC50 value as shown in Tables 3.3 and 3.4.

Species	96 hour LC50 (mg/L)	Water Hardness (mg/L)	Temp (°C)	pH	Reference
Banded kilifish	19.1	53	17	7.8	(Rehwoldt, *et al.*,1971)
White perch	14.3	53	17	7.8	(Rehwoldt, *et al.*,1971)
Carp	17	-	22	7.6	Present study
Tilapia	16.5	-	22	7.6	Present study

Table 3.3 96 hour LC50 values for zinc toxicity in freshwater fish.

Species	96 hour LC50 (mg/L)	Water Hardness (mg/L)	Temp. (°C)	pH	Reference
Fathead minnow	0.46-0.49	200	20-26	7.9	(Pickering *et al.*,1966)
Rainbow trout	0.33	374	15	7.7	(Dixon *et al.*,1981)
Fantail darter	0.33-.392	-	20	-	(Lydy *et al.*,1988)
Carp	0.05	-	22	6.9	Present study
Tilapia	1.52	-	22	6.9	Present study

Table 3.4 96 hour LC50 values for copper toxicity in freshwater fish.

The toxicity of water-borne metals also depended on a wide variety of water quality factors such as pH (Lauren *et al.*,1986). Sexual differences also affected the metal toxicity in fish. Tsai *et al.* (1981) presented that an increased rate of toxic action in the female guppy, *Lebistes reticulatus,* relative to that of the male. To minimize the sexual differences in metal toxicity, all the tilapia at juvenile stage for 96 hour LC50 toxicity test was chosen.

To select suitable sublethal doses for exposure tests, LC50 of metals should be considered. In tilapia, 96 hours LC50 for copper and zinc were 1.52 mg/L and 16.5 mg/L respectively while in carp, these were 0.05 mg/L for copper and 17 mg/L for zinc. The sublethal concentration of metals chosen was lower than the 96 hours LC50 value and 10, 50 and 100 μg/L of copper and zinc were selected for exposure tests. Since no reported toxicity test for cadmium in tilapia and carp, the waterborne dose of cadmium was determined according to the report from Environmental Protection Department (EPD) (Environmental Protection Department,1995) which indicated similar cadmium concentrations (0.2 μg/L) in the water samples from Tai Wai and Fo Tan. In the exposure tests, juvenile tilapia were exposed to a little bit higher cadmium concentration than reported from EPD (Environmental Protection Department,1995).

3.4.2 MT mRNA Expression of Tilapia under Aqueous Exposure of Metals

The metal uptake in the internal organs through aqueous exposure is relatively slower than injection. If there is a positive correlation of MT levels with metal contents in the tissue, it would be expected that the induction of MT levels in the internal organs is slower in fish exposed to waterborne heavy metal than injection. This was because the tissues would give effective response when the metal contents reached particular levels. However, metals did not uptake to the same extent in different pathways. It was possible that waterborne metal absorption through gills was slower than injection of metal to the body. Therefore, the internal tissues might have taken up metals through injection and should express MT rapidly.

In the present study, juvenile tilapia and carp were chosen in the exposure experiments because gender-related variations in toxic responses might affect MT levels in fish tissues. At the time of spawning, male fish of rainbow trout had elevated MT levels (Olsson *et al.*,1987). There were also changes in the MT content of the ovaries in female rainbow trout (Olsson *et al.*,1987). However, according to the result of Perkins *et al.* (1997), there were concluded that no significant differences

between MT levels and the gender of channel catfish exposed to waterborne copper. From the result of Cuvin-Aralar (Cuvin-Aralar,1994), different strains of *Tilapia mossambica* had different tolerance to metals. Therefore, it was the best to select one strain for the exposure test to minimize the individual difference in response. In the study, the strain of tilapia and carp for exposure tests were the hybrid of *Tilapia niloticus* and *Tilapia aurea* and carp is *Cyprinus carpio* from Agriculture and Fisheries Department (AFD).

Temperature and pH values might have an effect on MT gene expression or gene expression in general. MT induction in climbing perch living at higher temperature was observed to be faster when exposed to waterborne cadmium (Chatterjee *et al.*,1986), because temperature increased body metabolism and consequently faster response resulted. Changes in the water pH also affect the uptake of metals in at least two ways: (1) by affecting metal speciation directly and (2) by its effect on the biological surface (Campbell *et al.*,1985). Borgmann (1983) suggested that copper became less toxic to fish when the pH fell because the protons began to compete with the ligands that were involved in copper transport. In the present experiments, the temperature for exposure was kept at about 22°C and pH value ranged from 7 to 8.

At higher doses of cadmium exposure, both tilapia and carp showed negative correlation between fold induction of MTmRNA level and time course in the liver and gills. In agreement with previous reports in catfish (Chatterjee *et al.*,1991), plaice (George,1989), and rat liver (Yoshikawa *et al.*,1982); induction of MT synthesis is not linearly related to cadmium dosage in liver after cadmium administration. At 10 μg/L, the fold induction in gills was relatively higher than that in the liver. It was possible that the gills was the primary organ for metals uptake which was chelated by MT or MT-like proteins to control free metal ions entry into internal organs. In contrast, there was no substantial increase in MT or MT mRNA in the gills of fish exposed to waterborne metals (Olsson *et al.*,1987). At week 3, liver MT mRNA

levels increased in tilapia exposed to 10, 50 μg/L of cadmium but not in 100 μg/L of cadmium.

Pre-exposure of fish to heavy metals was shown to protect the animal from subsequent exposure to higher toxic metal levels (Klaverkamp,1984; Chan *et al.*,1987). Unger *et al.* (1996) indicated that MT mRNA level increased during cadmium exposure in oysters preexposed to cadmium. It thus appeared that MT was involved in protecting the organisms against the toxic effects of heavy metals.

From our results, no significant fold induction of MT mRNA was obtained in the tissues of tilapia exposed to copper. There seemed to be other copper binding proteins involved in copper transport (Pelgrom *et al.*,1995). In fact, most of the copper entered to the gills was transported via the blood plasma. Several proteins such as albumin and 'transcuprein' in the blood were involved for transporting copper to liver which copper was bound to ceruloplasmin and then released into the blood and distributed to other tissues (Cousins,1985; Weiss *et al.*,1985). Ceruloplasmin was also found in the plasma of tilapia but dose-response relationship with copper have not been found yet (Pelgrom *et al.*,1995).

Gills are the major pathway for the uptake of waterborne zinc in fish (Spry *et al.*,1988; Renfro *et al.*,1975). The rate of zinc uptake and accumulation, however, were reduced in high calcium concentration in water (Spry *et al.*,1988). MT seemed to be involved in the intracellular zinc transfer. During metal exposure, the portion bound to MT often increased and more than 80% of cytosolic zinc was found to bound with MT in zinc exposed fish (Hogstrand *et al.*,1995; Kito *et al.*,1982). Also, MT was involved in regulation of zinc during the reproduction in fish. In rainbow trout, the hepatic MT content increased during sexual maturation of females (Olsson *et al.*,1987). From our results, it indicated that little correlation between fold induction of MT mRNA and time in liver and gills at low doses (10, 50 and 100 μg/L). According to Table 3.2, the waterborne zinc from control aquarium was 157.47 μg/L, which was a little bit higher than the highest doses (100 μg/L) of zinc aqueous

exposure. Therefore, no significant result was obtained at low doses of zinc exposure. However, high doses of zinc (0.4, 1 and 2 mg/L) at week 1 and 2 gave good correlation with hepatic MT mRNA (r=0.942 for week 1 and 0.974 for week 2) (Table 3.5) and these zinc concentrations was more close to the sublethal concentration for juvenile tilapia.

3.4.3 Normalization of the Signals of Northern Blot Analysis

In the aqueous exposure experiments, actin levels were used for normalization because there was endogenous level in the liver and gills. In injection experiments, however, actin was chosen for normalization of the hybridization signals in northern blot analysis because muscle and heart in common carp were shown to have two hybridization signals with the zebrafish actin cDNA probe and different tissues expressed actin mRNA in a highly variable extent (Chan,1998).

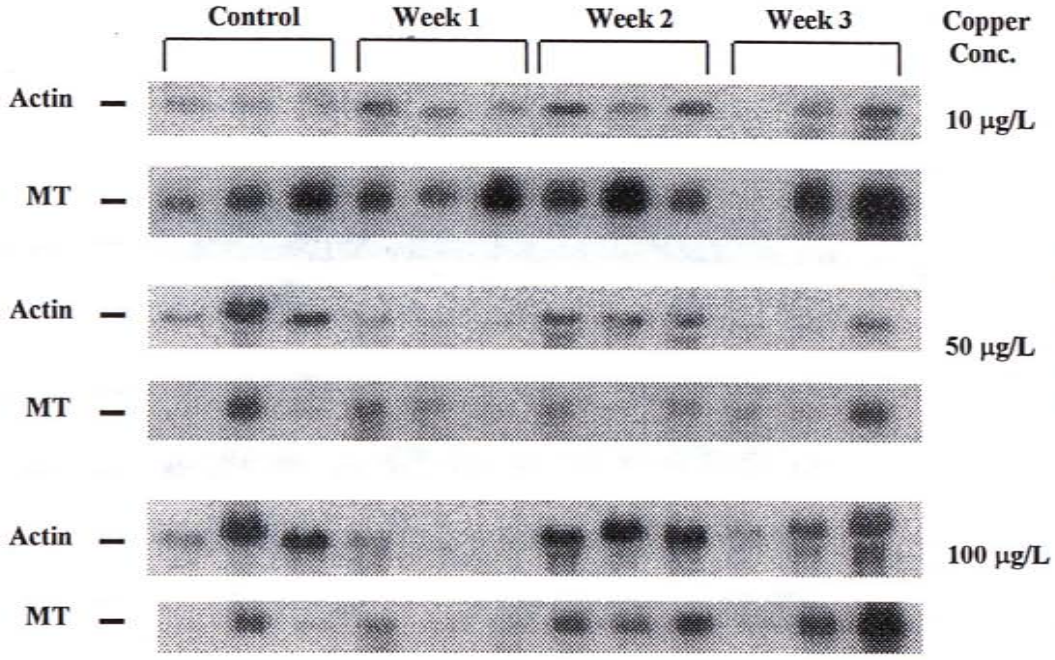
In fact, no probe is universally applicable for normalization for all tissues from all organisms. Therefore a proper positive control in northern blot analysis or RT-PCR should be chosen. The use of 18S ribosomal RNA (rRNA) level was a suitable positive control for normalization because 18S rRNA expression levels were similar in different tissues. However, as teleost 18S rRNA probe was not available in this laboratory, the signals from the 18S rRNA from the negative film of the agarose gels were used instead.

3.5 Conclusions

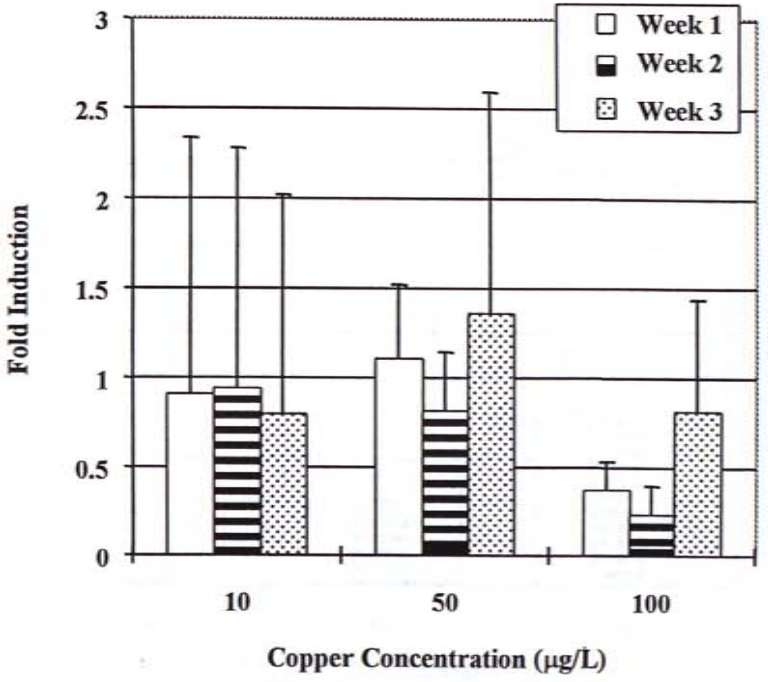
From the present study, the 96 hour LC50 values of zinc for tilapia and carp were similar but in 96 hour LC50 values for copper, tilapia was much higher than carp. In aqueous exposure experiments, there was positive correlation between fold induction of MT mRNA and time course at lower doses of cadmium and zinc. At 100 μg/L of cadmium and zinc in the liver of tilapia, however, negative correlation was obtained. At week 2, the higher doses of zinc (0.4, 1 and 2 mg/L) gave direct correlation with fold induction of MT mRNA levels in liver and gills of tilapia. Comparing gills with liver, there was lower fold induction of MT mRNA at 10 mg/L in the liver ($p<0.05$).

Conc.	Tilapia					
	Copper		Zinc		Cadium	
(μg/L)	Liver	Gills	Liver*	Gills*	Liver**	Gills**
10	-0.051	0.876	0.816	0.023	0.540	0.998
50	0.464	0.842	0.978	0.875	0.813	0.717
100	0.726	-0.862	-0.754	0.627	-0.559	0.831

Table 3.5 The summary of correlation between fold induction of MT mRNA levels in the tissues of tilapia and exposure time at different doses of metals. "*" and "" represents $p < 0.05$ and $p< 0.01$ respectively.**



Panel A



Panel B

Fig. 3.1 Northern Blot Analysis of MT mRNA in the liver of Tilapias exposed to copper 10, 50 and 100 µg/L at Week 1, 2 and 3. Panel A shows the result of the northern blot hybridized with actin and MT cDNA probes. **Panel B** The graph indicates the fold induction of MT mRNA which normalized by actin probe against time course at different copper concentrations.

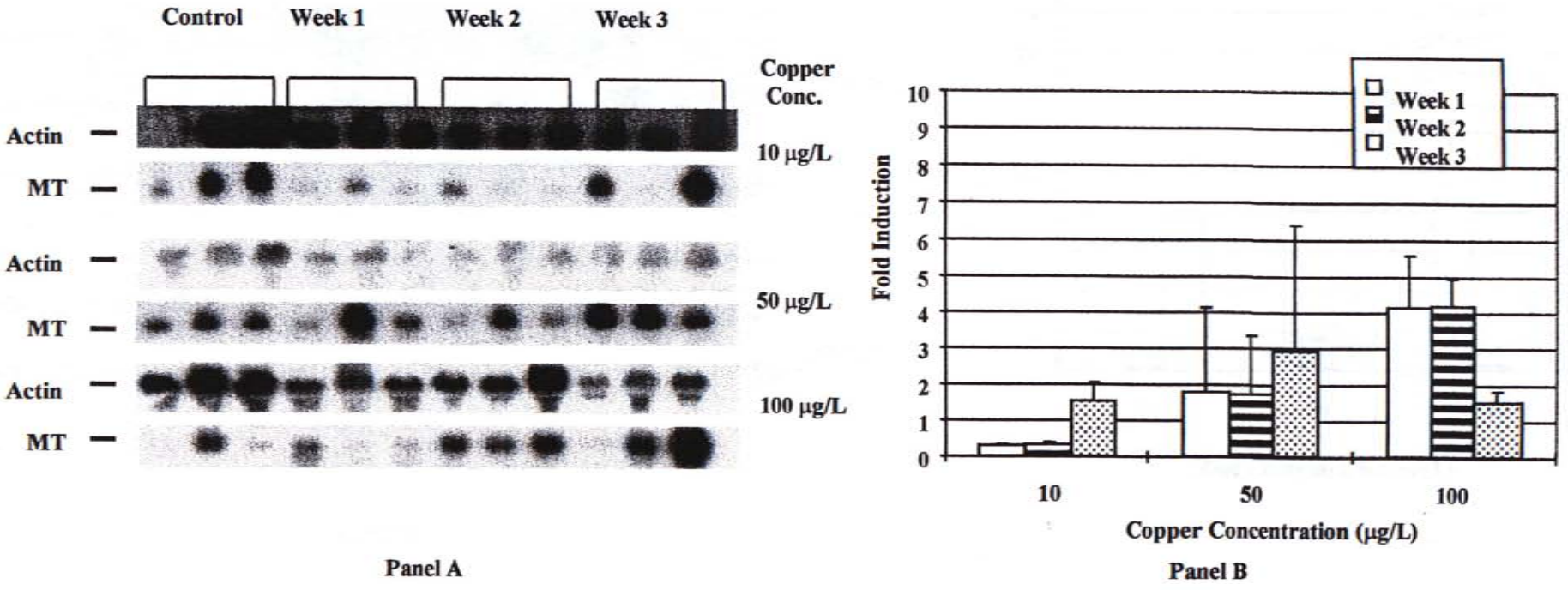
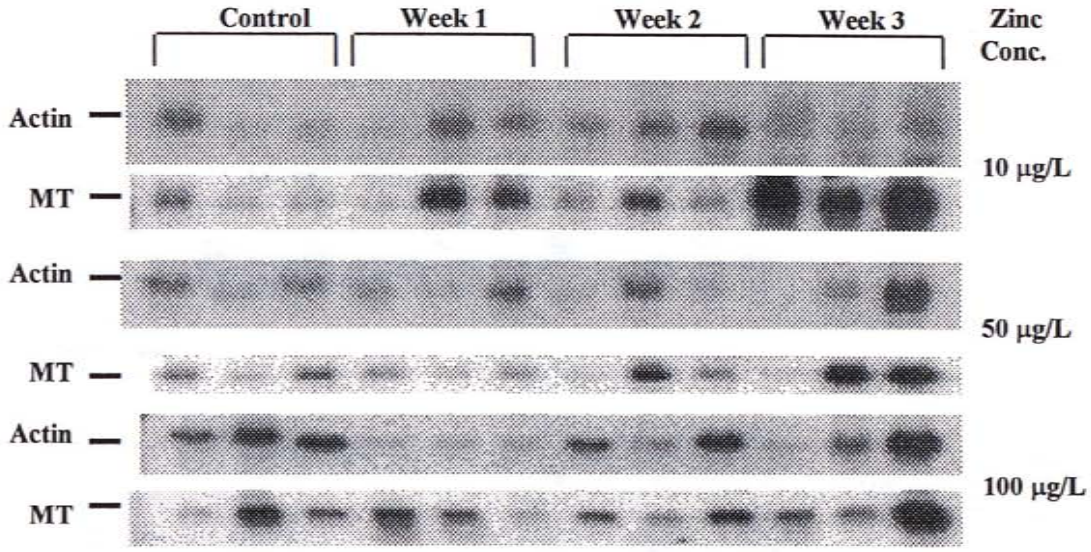
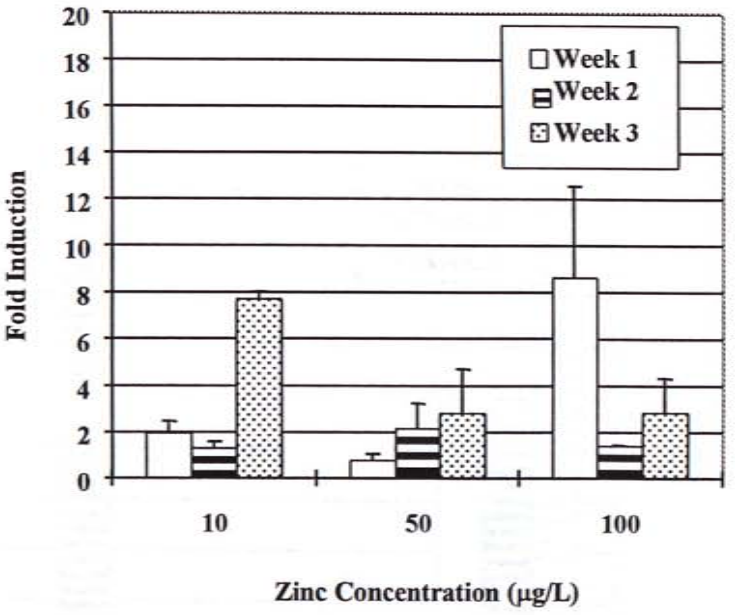


Fig. 3.2 Northern Blot Analysis of MT mRNA in the gills of tilapia exposed to copper 10, 50 and 100 µg/L at Week 1, 2 and 3. Panel A shows the result of the northern blot hybridized with actin and MT cDNA probes. **Panel B** The graph indicates the fold induction of MT mRNA which normalized by actin probe against time course at different copper concentrations.



Panel A



Panel B

Fig. 3.3 Northern Blot Analysis of MT mRNA in the liver of tilapia exposed to zinc 10, 50 and 100 µg/L at Week 1, 2 and 3. **Panel A** shows the result of the northern blot hybridized with actin and MT cDNA probes. **Panel B** The graph indicates the fold induction of MT mRNA which normalized by actin probe against time course at different zinc concentrations.

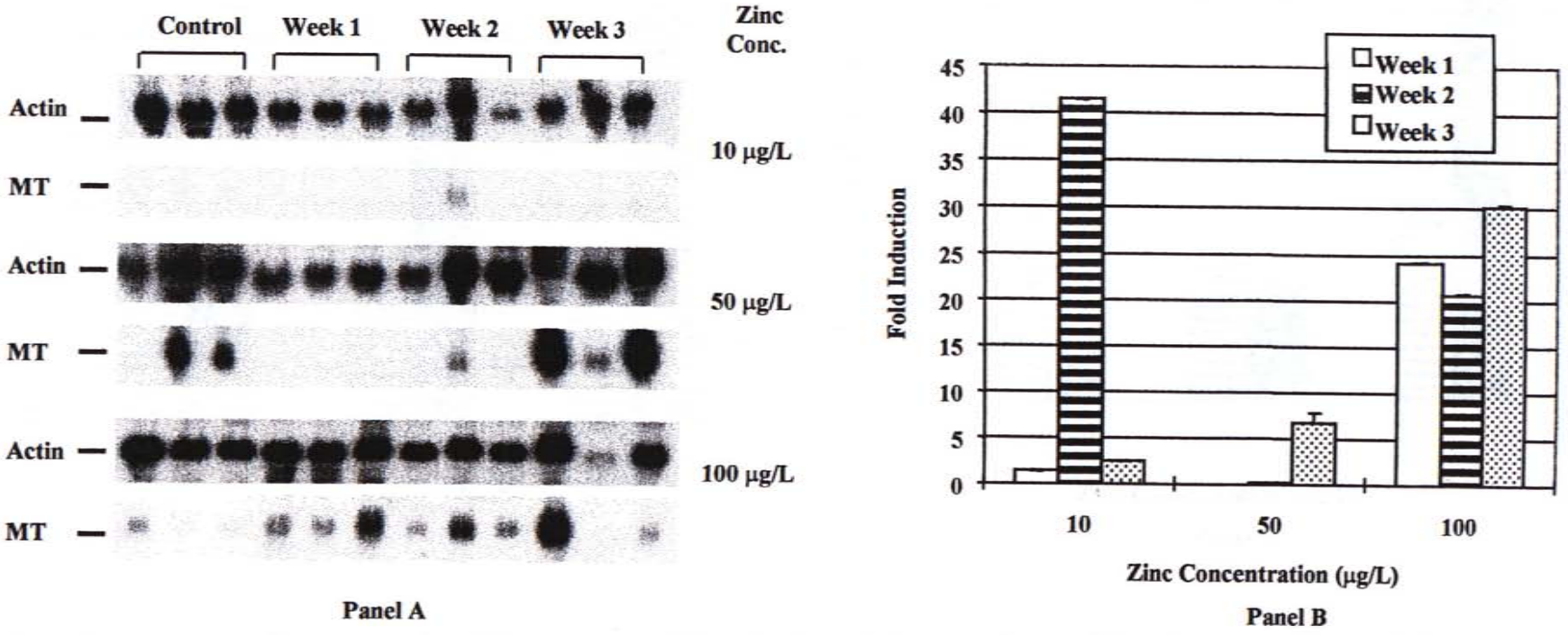


Fig. 3.4 Northern Blot Analysis of MT mRNA in the gills of tilapia exposed to zinc 10, 50 and 100 µg/L at Week 1, 2 and 3. **Panel A** shows the result of the northern blot hybridized with actin and MT cDNA probes. **Panel B** The graph indicates the fold induction of MT mRNA which normalized by actin probe against time course at different zinc concentrations.

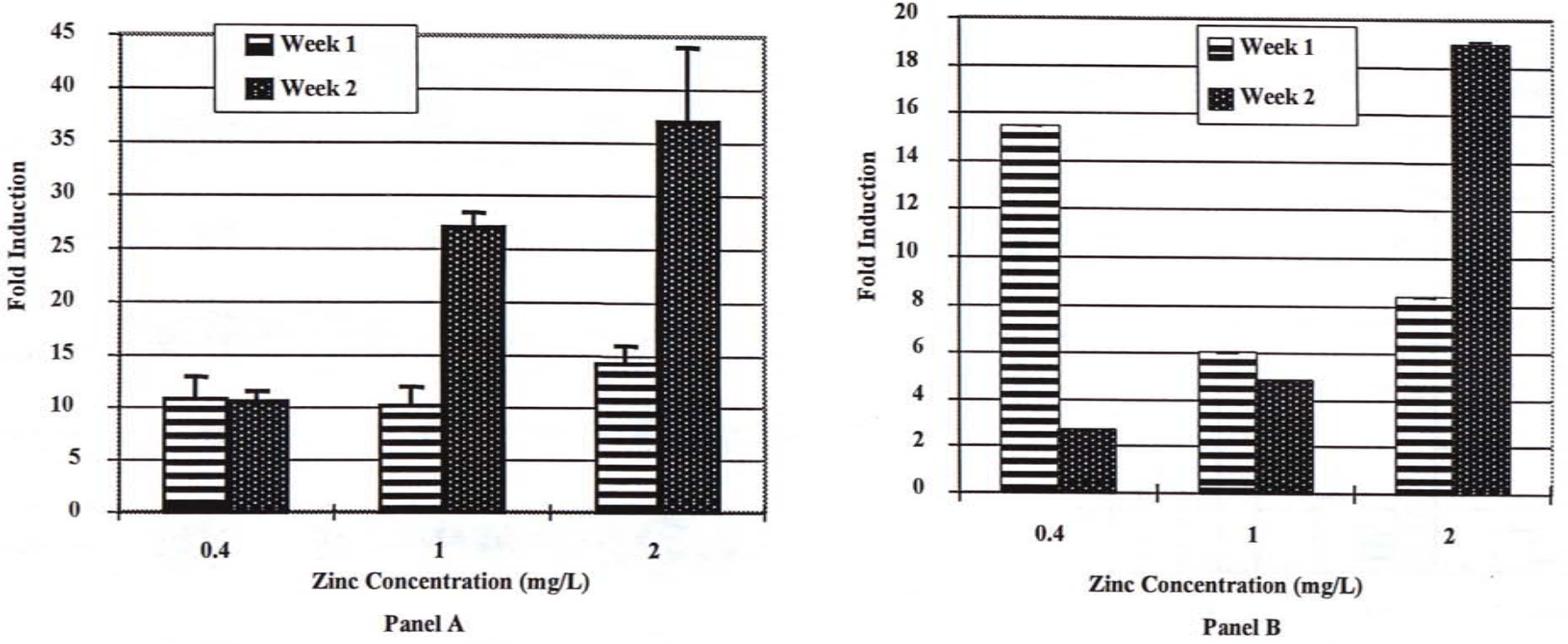


Fig.3.5 Northern Blot Analysis of Fold Induction of MT mRNA in the liver and gills of Tilapia exposed to zinc 0.4, 1 and 2 mg/L at Week 1 and 2. Panel A and Panel B showed the graph of fold induction of MT mRNA in the liver and gills respectively which normalized by actin probe against time course at different zinc concentrations.

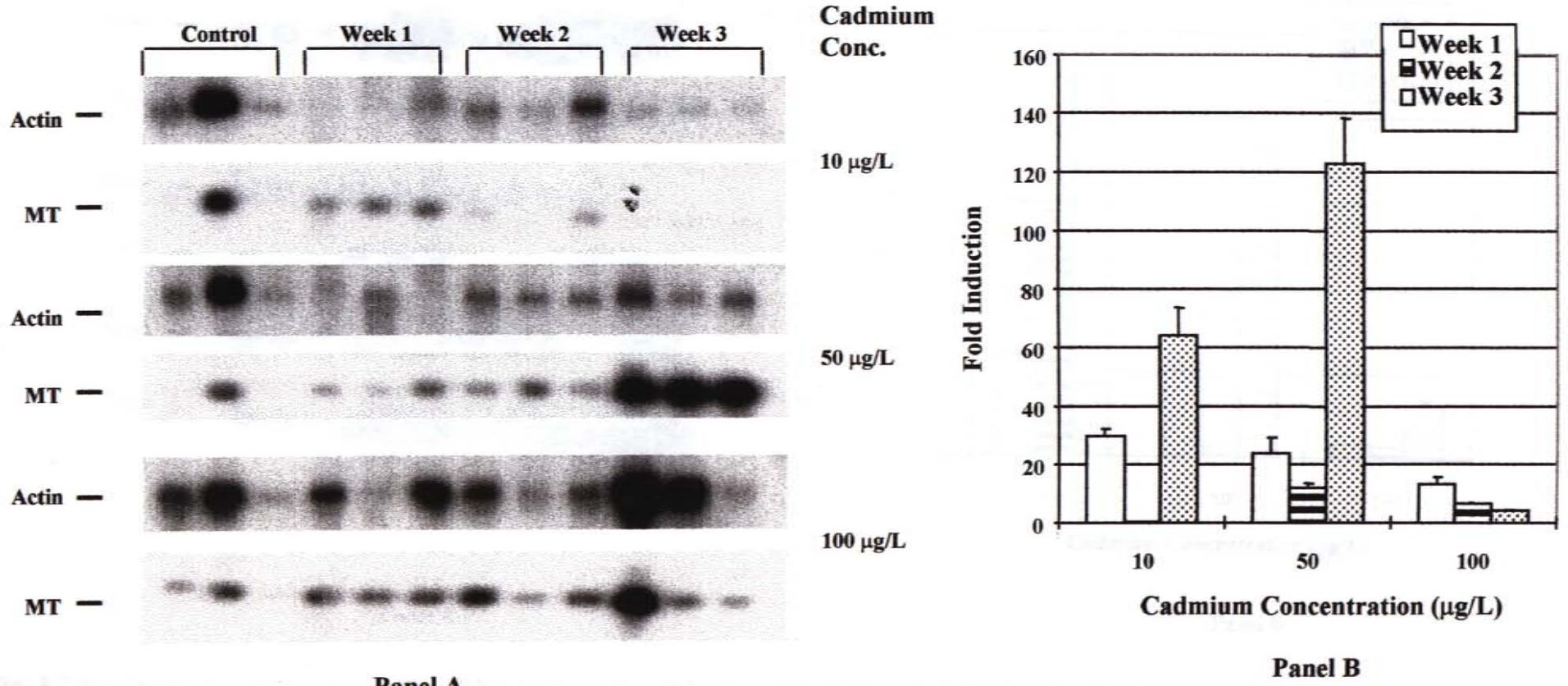


Fig. 3.6 Northern Blot Analysis of MT mRNA in the liver of tilapia exposed to cadmium 10, 50 and 100 µg/L at Week 1, 2 and 3. Panel A shows the result of the Northern blot hybridized with actin and MT cDNA probes. **Panel B** The graph indicates the fold induction of MT mRNA which normalized by actin probe against time course at different cadmium concentrations.

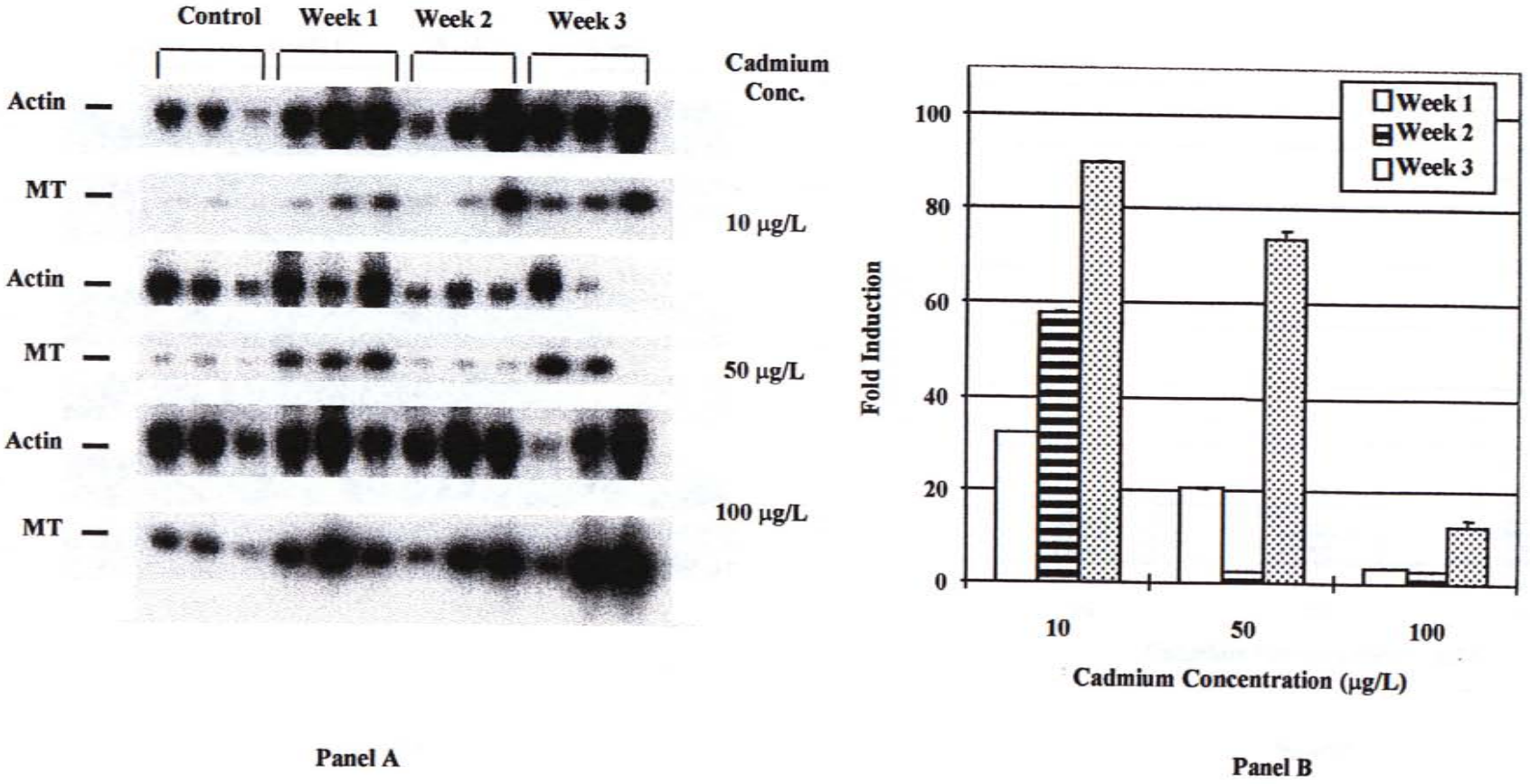


Fig. 3.7 Northern Blot Analysis of MT mRNA in the gills of tilapia exposed to cadmium 10, 50 and 100 µg/L at Week 1, 2 and 3. **Panel A** shows the result of the Northern blot hybridized with actin and MT cDNA probes. **Panel B** The graph indicates the fold induction of MT mRNA which normalized by actin probe against time course at different cadmium concentrations.

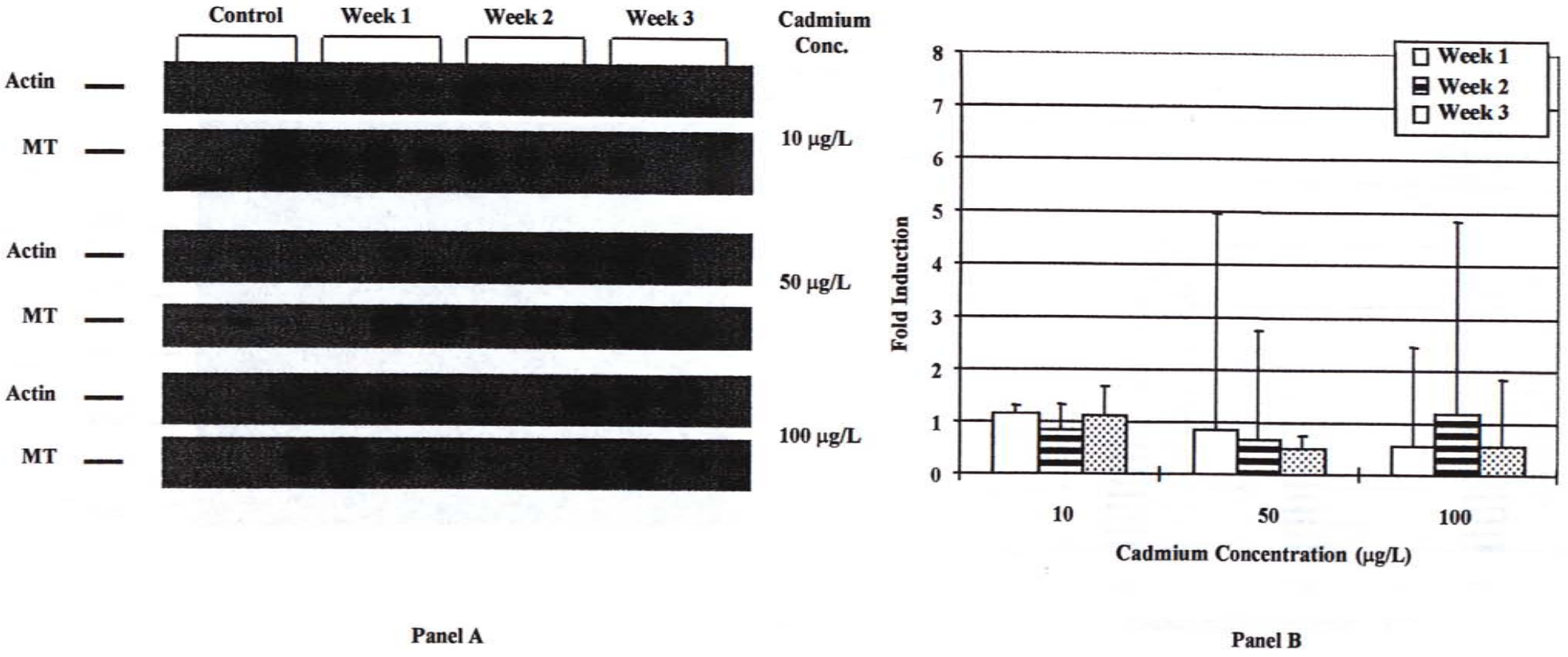


Fig. 3.8 Northern Blot Analysis of Fold Induction of MT mRNA in the liver of carp exposed to cadmium 10, 50 and 100 µg/L at Week 1, 2 and 3. Panel A shows the result of the northern blot hybridized with actin and MT cDNA probes. **Panel B** The graph indicates the fold induction of MT mRNA which normalized by actin probe against time course at different cadmium concentrations.

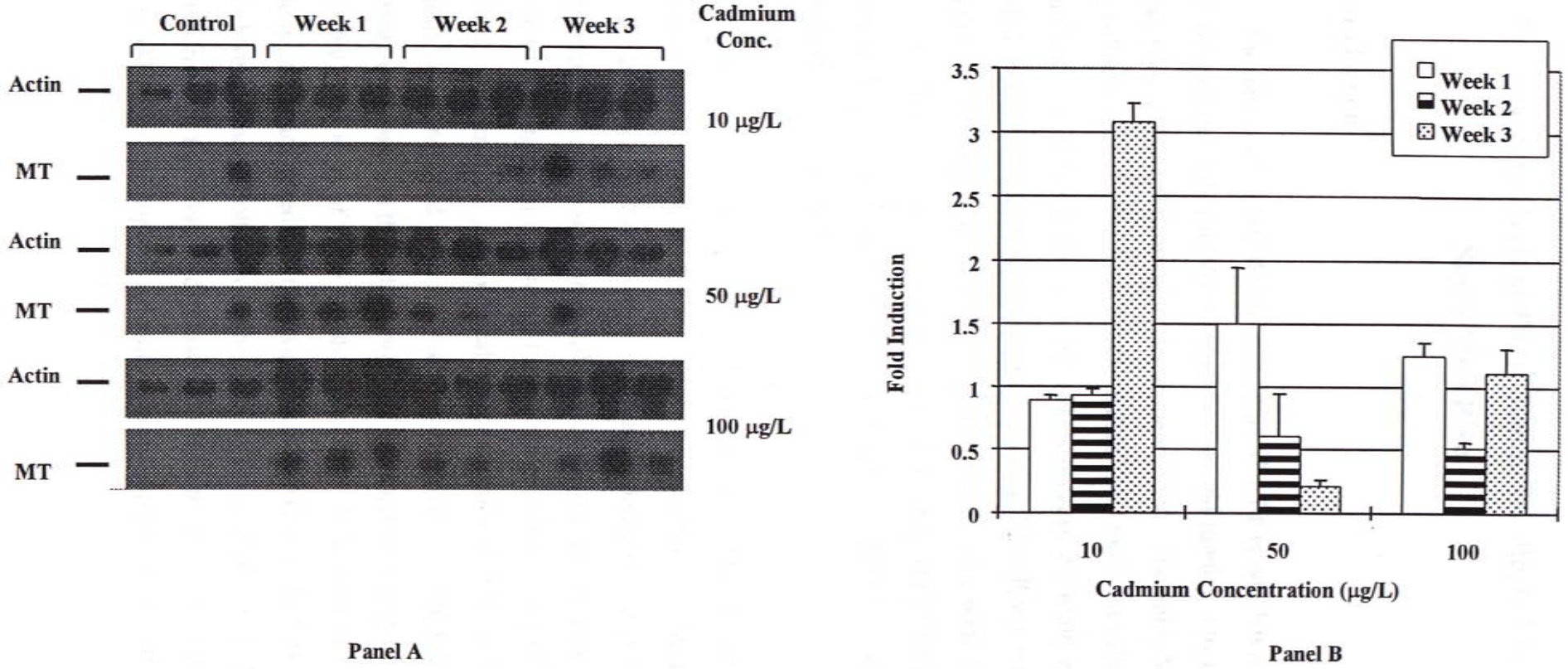


Fig. 3.9 Northern Blot Analysis of MT mRNA in the gills of carp exposed to cadmium 10, 50 and 100 µg/L at Week 1, 2 and 3. **Panel A** shows the result of the northern blot hybridized with actin and MT cDNA probes. **Panel B** The graph indicates the fold induction of MT mRNA which normalized by actin probe against time course at different cadmium concentrations.

Chapter 4 Analysis of Feral Samples Collected from Shing Mun River

4.1 Introduction

The field study was undertaken in Shing Mun River which is a 5 km river system running through Tai Wai, Shatin, Fo Tan and running into Inner Tolo Harbour (Environmental Protection Department,1995). The Shing Mun River, which collects water bodies from Fo Tan nullah, Siu Lek Yuen nullah and Tai Wai nullah were highly polluted in the 1980's because domestic wastes and industrial effluents were discharged into the Shing Mun River without any treatment. In addition, the situation was deteriorated by the poor turnover of water in the Shing Mun River due to its bottleneck topography (Environmental Protection Department,1995). The contaminants however are mainly accumulated in the sediments.

Surface water and sediments from the Shing Mun River contained elevated concentrations of metals including copper, cadmium, zinc, lead, nickel and chromium (Lee,1997). However, the measurements of metals in the water and sediment alone were sometimes difficult to link to aquatic organisms such as fish due to the migration of fish and fluctuation of water quality (Flemming *et al.*,1989). Research on physiological responses such as MT or MT mRNA synthesis in fish exposed to metals were investigated. In fish, preexposed to sublethal metals result in the induction of MT and can result in an acclimation to a potential toxic level of these metals in the water (Klaverkamp *et al.*,1984). Previous studies indicated that the level of hepatic MT in salmonids was related to the level of metal pollution in the waters (Roch *et al.*,1984; Roch,1986) whereas in perch and trout, studies also reported a positive correlation between hepatic MT levels and hepatic cadmium accumulation (Olsson *et al.*,1986).

Tilapias was chosen as a model fish as it is the most commonly found eurryhaline fish in local waters. From the results of a concurrent study by Lee (1997), the muscle and visera of tilapiaș accumulated metal ions, whether directly from the surrounding aqueous medium or indirectly via food chains.

4.1.1 Specific Aims of this Chapter

The aims of this chapter included:

1. Determination of metal concentrations (cadmium, chromium, copper, lead, nickel and zinc) in the tissues of tilapias collected from a field study.

2. Detection of MT mRNA levels in different tissues (gills and liver) of tilapias from the same field study.

3. Plot of the correlation between MT mRNA levels and metal contents in the tissues of feral tilapias.

4.2 Materials and Methods

4.2.1 Sampling Sites

There were two locations Fo Tan nullah and Tai Wai nullah in the Shing Mun River for samples collections as shown in Fig.4.1. In the Fo Tan nullah crossed at Shing Mun River, the samples were collected at the site near the "Hong Kong Sports Institute", whereas the samples were harvested at the Tai Wai nullah next to Man Lai Court (also linked immediately to Shing Mun River). Finally, the control tilapias were bought in the markets.

4.2.2 Data Analysis

Statistical analysis was performed with procedures in the Microsoft Excel package. The statistical analysis that was involved to interpret the results of metal contents and MT mRNA levels in the tissues of feral tilapias included one-way analysis of variance (ANOVA), and correlation analysis. Statistical significance was assessed at 5% significance level ($p \leq 0.05$).

4.2.3 Harvest of Feral Fish

Tilapia mossambica is the dominant fish species found in Shing Mun River. During the period of May 1996 and February 1997, about 35-40 feral tilapia were caught using gill net in these locations at mid-tide (between high and low tide). They were kept freshly in water-container and then transferred to the laboratory within 1 hour. Their weight and length ranged between 114 and 1000 g , 18.5-36 cm respectively. The feral tilapia were dissected and liver, gill and muscle were put in small vials, frozen in liquid nitrogen immediately and stored at -20°C (for acid digestion) or -80°C (for RNA extraction).

4.2.4 Determination of Metal Concentrations in the Tissues of Feral tilapia

All glassware, plastic bottles and containers used in acid digestion were washed and acid-treated with 0.5% of nitric acid overnight. The acid treated apparatus then were washed twice with Milli-Q water and dried at oven before used. The feral tilapias were dissected to have their gills, liver and muscle. Two grams of tilapias tissues were weighed and put into digestion tubes immediately. Five ml of 65% nitric acid and 2ml of 70% perchloric acid were added carefully. Glass funnels were placed on the digestion tubes and left the samples at room temperature for 2-4 hours and then placed the tubes on the heating block and heated at 115°C overnight (about 16 hours). This step is the pre-digestion step. After the funnels were removed, the samples were heated at 120°C for 4 hours.

Heating the samples to 125-140°C until they left around 0.5-1 ml of solution. The digested samples were cooled and then transferred into 50 ml volumetric flasks to make up to 50 ml with Milli-Q water. Finally, the diluted samples were filtered through ashless Whatman filter paper, transferred into plastic containers and stored at 4°C.

The metal concentrations of the samples prepared were measured by either flame SpectrAA-10 (Varian) (for metals with mg/kg levels such as copper and zinc) or Zeeman SpectrAA-800 (Varian) (for metals with μg/kg levels such as cadmium, nickel, lead and chromium). Standard solutions for different metals were prepared for setting calibration curves. The samples should be diluted if the metal concentrations were out of the calibration standards. Some modifiers were used for facilitating the measurement such as lead, 1% ammonium dihydrogen phosphate was employed during measurement. A reference material (Standard Reference Material 1566a Oyster Tissue) and blank (acid digestion without tissues) were used in each block of tubes gone through acid digestion for the verification of the digestion process.

4.2.5 Endogenous MT mRNA level using Northern Blot Analysis

Refer to Section 2.2

4.2.6 Calculation of MT mRNA levels and Analysis of Results

Refer to 2.2.2.6 except using actin probe insteading of 18S rRNA for normalization

4.3 Results

4.3.1 Metal Contents in the Tissues of Feral Tilapia

The metal concentrations in different tissues of feral tilapias from different sites were presented in Table 4.1. The results indicated that different tissues have various concentrations of heavy metals. For example, muscle and gills showed much lower levels of metal ions than that in the liver. Copper and chromium concentrations in the gills of control fish were slightly higher than the other (Fig.4.2). However, Tai Wai and Fo Tan were about 2-fold and 5-fold higher nickel concentration than that in the control fish, respectively. Apart from that, the cadmium concentrations in the gills of tilapias from Fo Tan (47.07μg/kg) were a little bit higher than those from Tai Wai (30.01μg/kg).

All the sites had similar chromium concentration in the liver (Fig.4.3a). Nickel concentrations in the liver of tilapia from Tai Wai and Fo Tan were 9-fold and 4.5-fold higher than that of the controls respectively (Fig.4.3b). Furthermore, control liver showed less cadmium and lead concentrations than those from Fo Tan (2.8 and 2.9 fold respectively) and Tai Wai (2.8 and 4 fold respectively). Control liver gave similar copper concentration with Fo Tan, which were 2-fold lower than that of the tilapias from Tai Wai. There were no significant differences in zinc concentrations of tilapias tissues from all sites.

4.3.2 Comparison of Metal Concentrations Among different Tissues of Feral Tilapias

Copper concentrations in the liver were significantly higher than that of the gills and muscle. Similar pattern was noted for zinc (Fig.4.2b) and cadmium concentrations (Fig.4.2c). The cadmium concentrations in the gills and muscle were below 50 μg/kg, whereas cadmium concentration in the liver was about 6-fold higher than that of the gills and muscle. From Fig.4.3b, tilapias from Tai

Wai and Fo Tan showed similar trends of their nickel concentrations in different tissues (liver>gills>muscle), but in control there were approximately the same among different tissues. The chromium concentrations among tissues were approximately the same in different sites except in control, which had higher chromium concentrations in the gills (Fig.4.3a). Tilapias of Tai Wai and Fo Tan also gave higher lead concentrations in the gills than liver (Fig.4.3c).

4.3.3 MT mRNA levels in the Tissues of Feral Fish

The results of northern blot analysis of MT mRNA levels in the gills of tilapias from Tai Wai and Fo Tan are shown in Figs. 4.4 to 4.7. The MT mRNA levels in the gills of Tai Wai were found to be higher than Fo Tan (around 10-fold higher) and mainly existed during the summer. There was no detectable MT mRNA levels in the gills of controls. The liver MT mRNA levels of tilapia from Tai Wai also gave higher MT mRNA levels than those from Fo Tan and control.

4.3.4 Correlation Between Metal Contents and Endogenous MT mRNA Levels in the Tissues of Feral Fish

Negative correlations between MT mRNA levels and cadmium or chromium concentrations in the liver of tilapias from Fo Tan were found (Fig.4.8a and Fig.4.8c). Fig. 4.8b showed that there were slight increases of MT mRNA level with increasing nickel concentration. However, MT mRNA levels decreased with elevation of cadmium concentrations in Fig.4.9a and 4.9c. There was little correlation between MT mRNA levels and zinc concentrations shown in Fig. 4.9b. In Fig. 4.10 and 4.11, MT mRNA levels had no correlation with metal concentration in the gill except cadmium concentrations (Fig.4.11b) which showed high positive correlation with MT mRNA levels ($r=0.636$) (Table 4.2).

From Figs. 4.12 and 4.13, hepatic MT mRNA levels in the Tai Wai's liver showed positive correlation with all the metals especially lead and nickel, which gave higher correlation coefficient value (r=0.402, 0.467 respectively) (Table 4.2). In the gills, the relationship between metal concentrations and MT mRNA levels were quite different. Lead, zinc and copper showed not strong correlation while nickel and cadmium gave slight positive correlation in Fo Tan gills (Figs.4.14 and 4.15). There was little correlation between the MT mRNA levels and chromium in the gills showed in Fig.4.14c (r=0.471) (Table 4.2).

4.4 Discussion

4.4.1 Bioaccumulation of Metals

In the present study, these six heavy metals were investigated because these metals are widely present in the local water such as Shing Mun River which are highly polluted by industrial effluents. On the other hand, the highest metal contents in the tissues of feral tilapias are found in the liver, which is the most important organ for metal detoxification (Buckley *et al.*,1982). Lauren *et al.* (1988) reported that at least 40% of the whole body copper was presented in the liver in rainbow trout during metal exposures. In mammals, *in vitro* studies showed that the hepatic copper binded with low-molecular weight cytosolic ligands such as MT (Bremner,1987) and accumulated in hepatocytes (Linder *et al.*,1996). The livers reduced hepatic copper concentration either by retaining in subcellular particles such as nuclei and mitochondria (Salmenpra *et al.*,1986), excreting into the bile (Davis *et al.*,1987) or releasing back into the carrier proteins in plasma. However, this might not always true for all metals. From the result of Olsson *et al.* (1996), the kidney of rainbow trout usually accumulated considerably more cadmium than the liver. Therefore, metal accumulation could be tissues specific and species specific.

In the result of this study, metal concentrations in gills were relatively lower than liver. Similar results were obtained from Farag *et al.* (1995), which showed that the copper concentration was lower in the gills than liver of brown

trout. However, gills were the primary sites for soluble metals absorption from aquatic environment. Soluble metals passed through the gills and transferred into the circulatory system by binding with some unknown carrier proteins (Percival *et al.*,1990).

From the present study, the concentrations of metals measured in the muscles were much lower than that of other tissues. Comparing the results with those from Lee (1997), feral tilapias muscles showed similar metal concentrations except zinc which was significant higher in the present study. Chan (1995) reported that metal concentrations (all in dry weight) in the muscle of rabbitfish were 5.7±4.0 mg/L for copper, 66.6±31.8 mg/L for zinc, 3.3±1.1 mg/L for cadmium and 19.1±6.3 mg/L for lead. In comparison with other local or vicinity data published (Luk *et al.*,1990; Phillips *et al.*,1982), however, metal concentrations were relatively low in the present study (all in wet weight) (11.76 (11.58) mg/L, 0.265 (0.35) mg/L, 9.74 (6.84) μg/L and 132 (210) μg/L for zinc, copper, cadmium and lead respectively in Tai Wai and Fo Tan.

There is another primary organ for soluble metal uptake, such as intestine, which has not been investigated in our study. Lee (1997) also reported that the viscera of feral tilapias from Shing Mun River possessed high metal contents. Dietary metals administered into the internal organs through gastrointestinal system raised the metal content in intestine increase. This exogenous level of metals stimulated their absorption. For instance, as well as in mammal, zinc was complexed in the intestinal mucus of the upper part of small intestine in winter flounder (Shears *et al.*,1983; 1979) before it entered the mucosal cells by an unknown process. After entering the mucosal cells, zinc was complexed with low molecular weight metal binding proteins such as MT (Shears *et al.*,1979; Weber *et al.*,1992). Since no basolateral carrier has been found in the mucosal cells of fish, the transfer of zinc into the blood might be a passive process (Shears *et al.*,1983).

In the present study, the concentrations of zinc, copper, nickel and lead were higher in the livers of adult feral tilapias collected in 1996-97 from Tai Wai than in those of adult of feral tilapias from Fo Tan. The most elevated concentrations of these four metals were copper which was approximate double to the other sites similar to the results of the water or sediments reported from EPD (Environmental Protection Department,1995). The copper concentration in water sample from Tai Wai was 10 μg/L higher than Fo Tan (22 μg/L). However, the report showed zinc and lead contents in water sample from Tai Wai were 60 μg/L and 2 μg/L respectively which were lower than from Fo Tan. Comparing with the result from Lee (1997), water samples from Tai Wai were similar with metal concentrations from Fo Tan.

Sediment metal concentrations had no significant difference among the two sites except lead and nickel which showed 0.5 and 2-fold higher in Tai Wai than Fo Tan. It seemed to be possible that there was a relationship between hepatic and sediment nickel concentration. Comparing the metal contents in feral tilapias with those in barnacles from Ma Liu Shui by Rainbow *et al.* (1992), the metal contents in feral tilapias were lower than in barnacles, which are filter feeder and can accumulate metal ions (1808 mg/L for copper, 14818 mg/L for zinc and 22.1 mg/L for cadmium in the barnacles). Since the barnacles could accumulate high level of metals, this seemed to be a good biological indicator for metal pollution. There is still obscure that feral tilapias is good biological indicator but caged fish with genetically similar tilapias might be a better way for monitoring metal pollution in fish.

4.4.2 Endogenous level of MT mRNA in the feral fish

In conjunction with the significantly higher metal concentrations in livers of feral tilapia from Tai Wai than the control, the endogenous level of MT mRNA in livers and gills were estimated by northern blot analysis. Generally speaking, hepatic MT mRNA level was higher in Tai Wai than Fo Tan.

MT is likely to be associated with the regulation of the intracellular pool of free trace metals. When the exogenous metals increase, MT or MT mRNA could be elevated to reduce the metal toxicity. Extensive studies were investigated on the induction of MT against the dose of metal administration. Injection of copper and zinc all resulted in an elevation of MT mRNA in winter flounder and salmonids (Zafarullah *et al.*,1989; Chan *et al.*,1989). During 5 days exposure of rainbow trout to waterborne zinc, an elevated level of MT was found in the liver (Brady,1982). Hogstrand *et al.* (1996) reported that liver of squirrelfish contained remarkably high concentrations of MT and zinc.

Because MT is inducible by metal residues, MT was proposed as biomarker of exposure to monitor the pollution level of metal in aquatic environment. In Vancouver Island of Canada, MT levels in livers of rainbow trout obtained from lakes and rivers with different degrees of metal pollution was significant correlated with zinc contents (Roch *et al.*,1984). The data from Sulaiman *et al.* (1991) indicated that MT levels in livers of winter flounder caught at various stations along the River Forth Estuary in Scotland were an accurate reflection of sedimentary metal levels and metal bioavailability in livers. Olsson *et al.* (1986) reported that there was positive correlation ($r=0.84$) between metallothionein and subcellular cadmium concentration in the liver.

From the present result, no significant correlation of MT mRNA and metal contents in the tissues of feral tilapias was obtained. However, cadmium and MT mRNA levels in the gills of tilapias from Fo Tan showed some degree of

correlation (r=0.64). Apart from metal as inducer for MT gene expression, several factors can induce the MT levels such as hormones (Waalkes *et al.*,1990) and starvation (Overnell *et al.*,1986). Moreover, the individual differences among the feral fish were quite higher in feral tilapias.

From the result of our laboratory (Shen,1998), the metal concentration was analyzed for their distribution in subcellular fractions of tilapia liver homogenate as showed in Table 4.2.

As shown in table 4.3, zinc was found to be more associated with the cytosolic fraction (105000 x g ultra-supernatant) (61%), whereas copper and cadmium were mainly associated with the 15,000 x g pellet, containing nucleus and membranes (58% for copper; 54% for cadmium). Therefore, other metal-binding proteins seemed to be produced to cope up with the elevation of metal ions simultaneously (Shen *et al.*,1998).

4.5 Conclusions

From the present study, metal concentrations especially copper in the tissues from Tai Wai were higher than Fo Tan. Furthermore, livers had the highest metal concentrations followed by the gills and muscles.. Elevated levels of hepatic copper were found in Tai Wai indicated that copper contamination in Tai Wai nullah was still a problem. Moreover, the endogenous hepatic MT mRNA level in Tai Wai was higher than those in Fo Tan. However, there was no significant correlation between metal content and MT mRNA level in the tissues of feral tilapia from Tai Wai and Fo Tan except gills with some correlation between cadmium concentrations and MT mRNA levels in tilapia from Fo Tan.

Table 4.1
MT mRNA levels and Concentrations (wet weight) of Zn, Cu, Cd, Ni, Pb, and Cr in tissues of Tilapia collected from Shing Mun river in 1996-97

		Muscle				Liver				Gills			
		n	Mean	SD	Range	n	Mean	SD	Range	n	Mean	SD	Range
Zn:	Tai Wai	35	9.74	6.01	nd,6-24.7	35	29.61**	12.77	12.5-58.9	35	18.627*	5.066	8.04-34.22
mg/kg	Fo Tan	40	11.58	5.54	4.69-27.26	40	21.69	9.03	11.5-45.4	40	17.76	4.84	9.196-34.8
	Control	28	6.7	0.89	5.1-8.68	28	21.7	2.42	16.1-25.3	28	14.8	1.76	9.4-19.48
Cu:	Tai Wai	35	0.22	0.14	nd,0.137-0.584	35	296.7**	154.3	16.5-828.1	35	1.1**	0.813	0.517-40
mg/kg	Fo Tan	40	0.35	0.27	0.137-1.37	40	175	159	56.3-1052	40	1.047	0.718	0.294-3.301
	Control	28	0.3	0.519	0.04-1.38	28	155.4	81.8	53.6-408.11	28	1.5	1.493	0.1-6.38
Cd:	Tai Wai	35	8.07	8.75	nd, 3-42.1	35	315	167	83-819	35	30.01	24.67	nd, 6-81.5
μg/kg	Fo Tan	40	6.86	5.32	0.46-21.1	40	317.5	243.6	79-921	40	47.068	64.489	3.9-188
	Control	28	8.8	6.8	3-25.66	28	110.5	53.0	43.14-2523	28	47	35.78	9-118.19
Ni:	Tai Wai	35	140	0.121	nd, 71-583	35	947**	0.409	352-1963	35	287**	0.129	361-2056
μg/kg	Fo Tan	40	200	0.150	65-572	40	468	0.278	165-1535	40	308	0.121	110-652
	Control	28	62.77	14.48	nd, 55-105	28	102	60.1	4-241	28	96.7	40.5	45-201
Pb:	Tai Wai	35	109	0.153	nd, 14-586	35	316*	0.202	nd, 100-790	35	491**	0.646	131-603
μg/kg	Fo Tan	40	215	0.32	53-1590	40	231	0.165	95-935	40	594	0.62	86-2989
	Control	28	60	79.6	nd, 3.19-302	28	79	57.9	nd, 3-236	28	99.1	102.52	nd, 0.42-502
Cr:	Tai Wai	35	160.7*	0.092	nd, 110-391	35	201*	0.08	114-419	35	141**	0.04	0.123-0.669
μg/kg	Fo Tan	40	153	0.057	83-394	40	193	0.085	92-463	40	162	0.055	0.073-0.318
	Control	28	169.1	179.56	nd, 5.49-302	28	185	0.185	32-674	28	259	259	0.013-0.986
MT mRNA	Tai Wai					35	4.25	8.15	nd, 0.0032-47.8	35	1.10	2.87	nd, 0.0584-12.88
	Fo Tan					40	3.28	8.10	0.0001-33.76	40	0.17	0.13	nd, 0.0273-0.472
	Control					28	0.91	0.92	nd, 0.0023-2.838	28	nd	nd	

*': $p < 0.05$;'**': $p < 0.01$ significant difference compared to Fo Tan (t-test). 'nd' means not detectable.

Metal	Tai Wai		Fo Tan	
	Gills	Liver	Gills	Liver
Lead	-0.188	0.403	0.0678	0.0529
Nickel	0.141	0.467	-0.144	0.124
Chromium	0.433	0.182	0.0026	-0.0088
Cadmium	0.192	0.348	0.636	-0.23
Zinc	-0.302	0.282	0.0787	0.273
Copper	-0.064	0.254	-0.173	0.064

Table 4.2 Correlation coefficients between metal concentrations and MT mRNA levels in the tissues of tilapia caught from Tai Wai and Fo Tan.

	Copper	Zinc	Cadmium
15000 g pellet (membranes, nuclear, etc.)	58	12	54
105000 g pellet (mic rosomal fraction)	36	27	14
105000 g supernatant (cytosolic fraction)	16	61	32

Table 4.3 Distributions (%) of copper, zinc and cadmium in subcellular fractions of tilapia liver homogenate (Shen *et al.*,1998).

Metals	Shing Mun River	
	Gills	Liver
Copper	0.1816	0.1668
Zinc	0.0933	0.283
Cadmium	0.05	0.0006
Chromium	0.4474	0.0349
Nickel	0.2581	0.2916
Lead	-0.0826	0.245

Table 4.4 Correlation coefficients of MT mRNA with metal concentrations in the tissues of Tilapia from Shing Mun River.

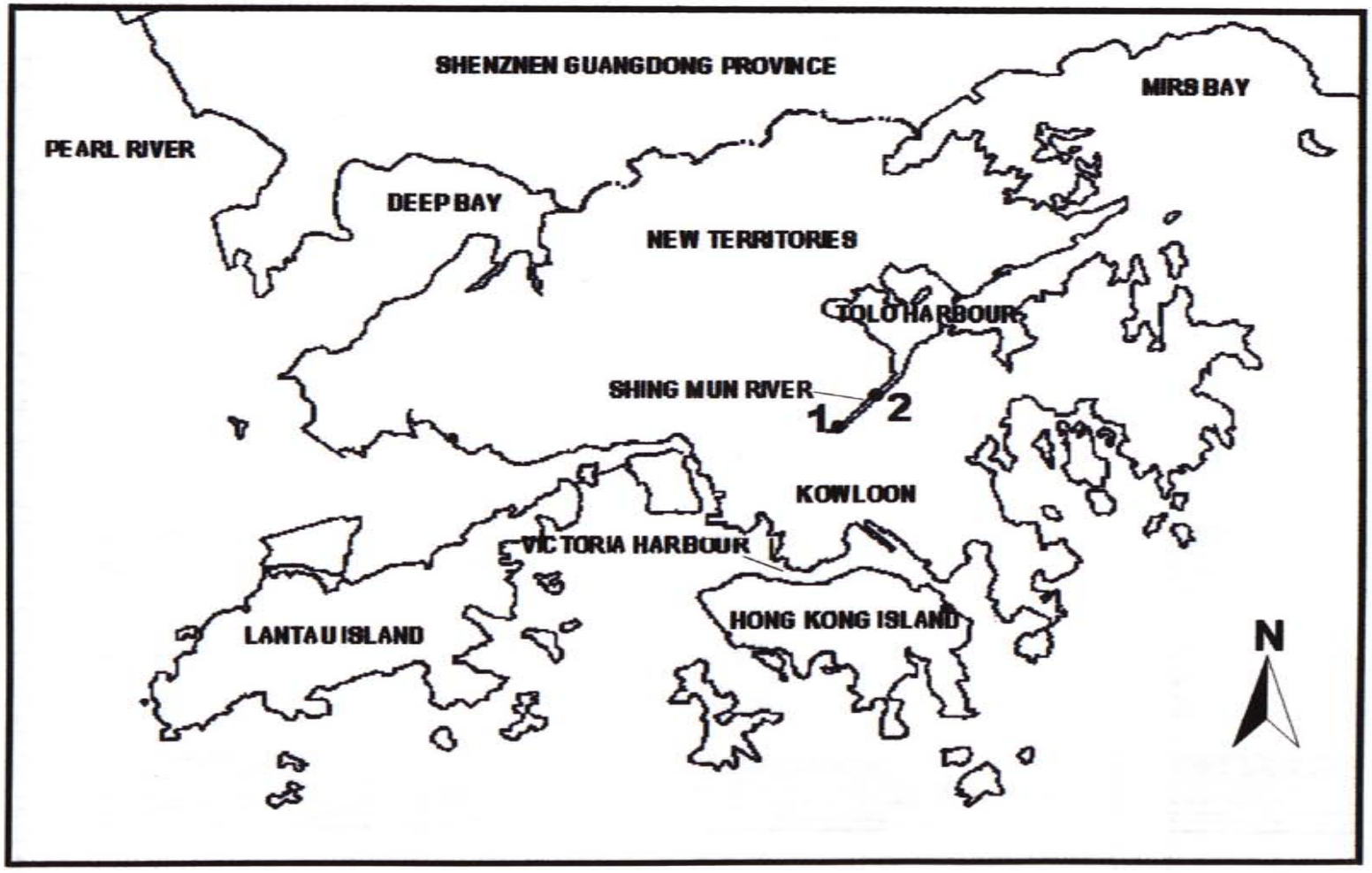


Fig.4.1 Sketch map of Hong Kong showing the sampling sites of Tai Wai (1), and Fo Tan (2) at Shing Mun River.

Fig. 4.2a

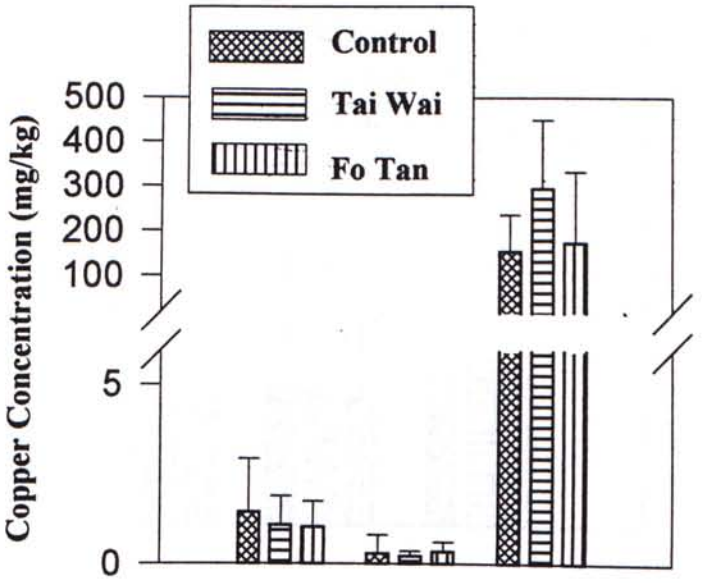


Fig. 4.2b

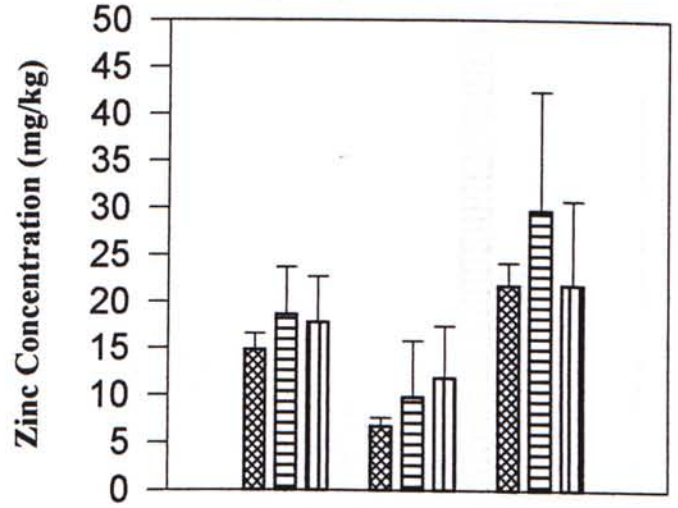


Fig. 4.2c

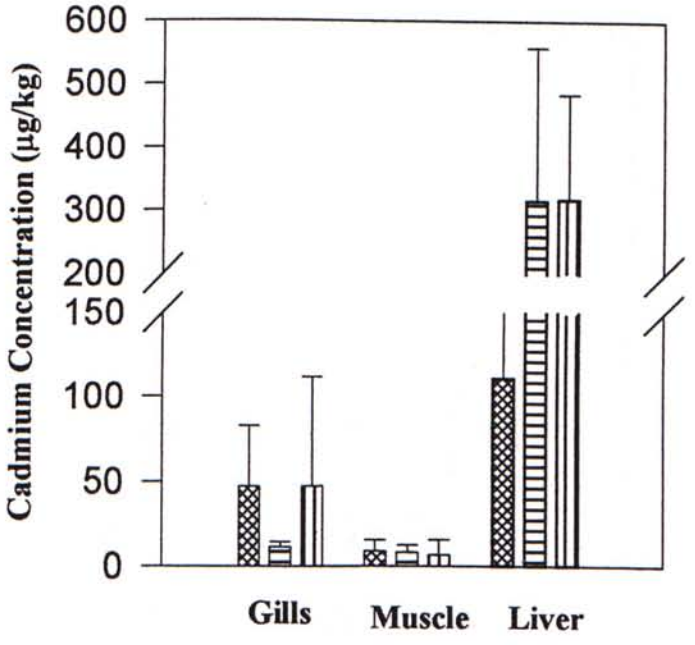


Fig.4.2a, b and c. Copper, zinc and cadmium concentrations (wet weight) in different tissues of tilapia from different sites.

Fig. 4.3a

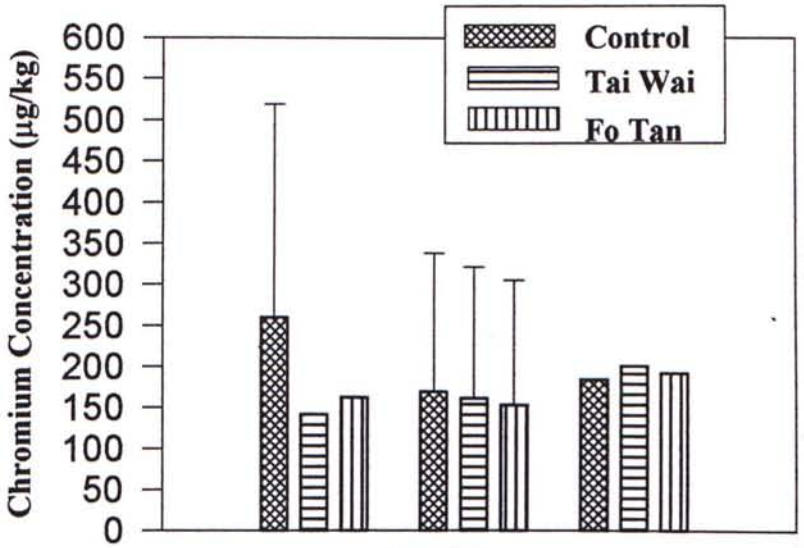


Fig. 4.3b

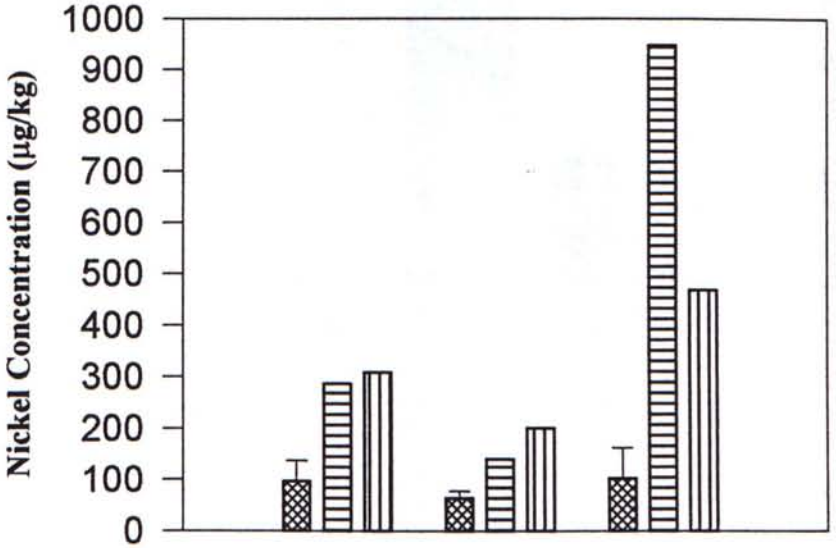


Fig. 4.3c

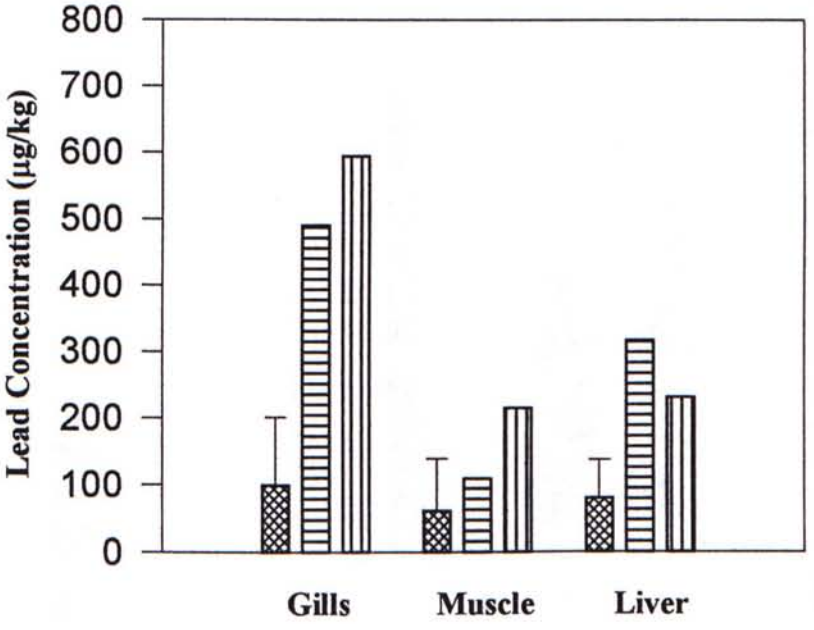


Fig.4.3a,b and c. Chromium, nickel and lead concentrations (wet weight) in different tissues of tilapia from different sites.

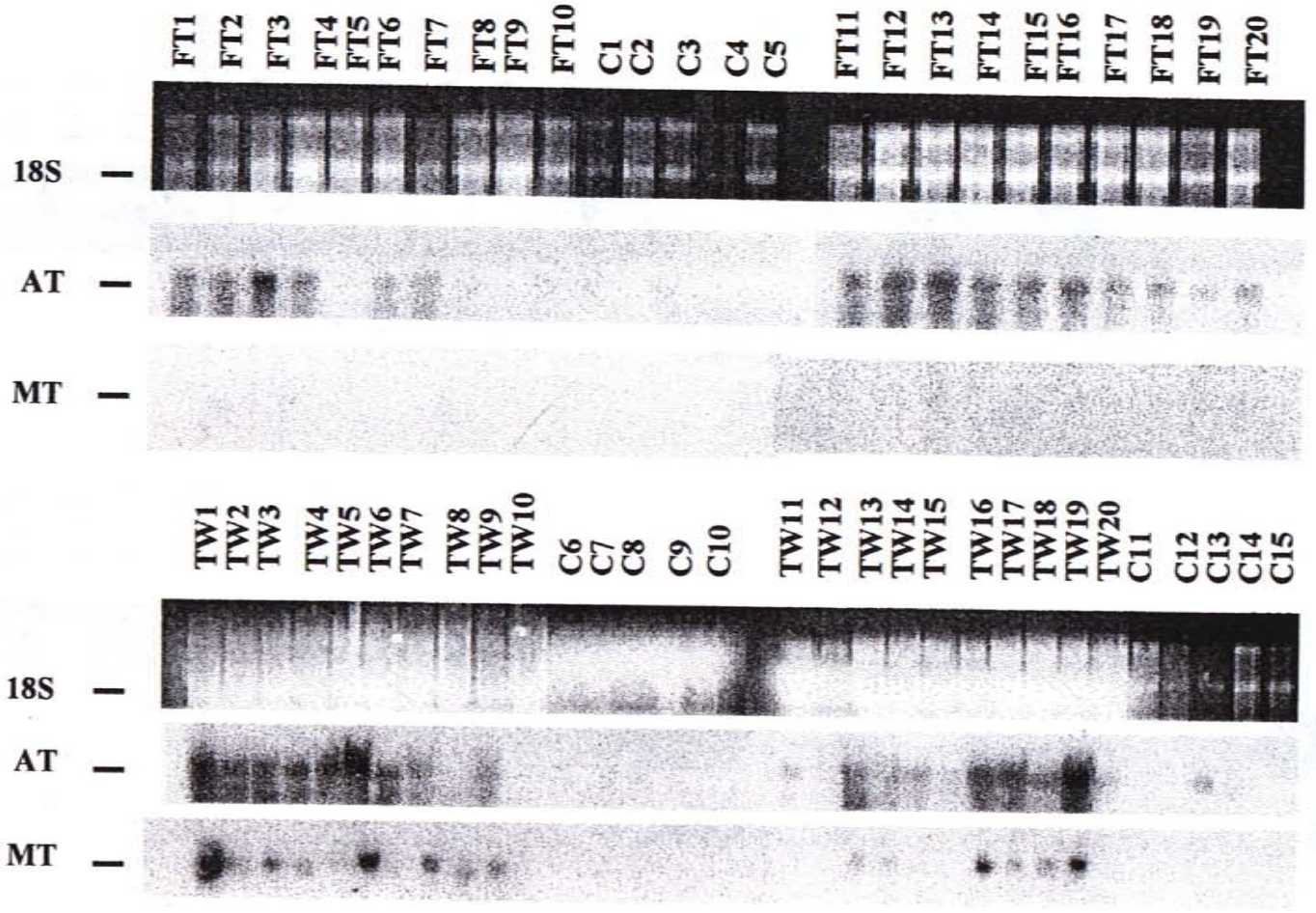


Fig. 4.4 Northern Blot Analysis of MT mRNA level in the liver of tilapia from different sites, Tai Wau (TW) and Fo Tan (FT).

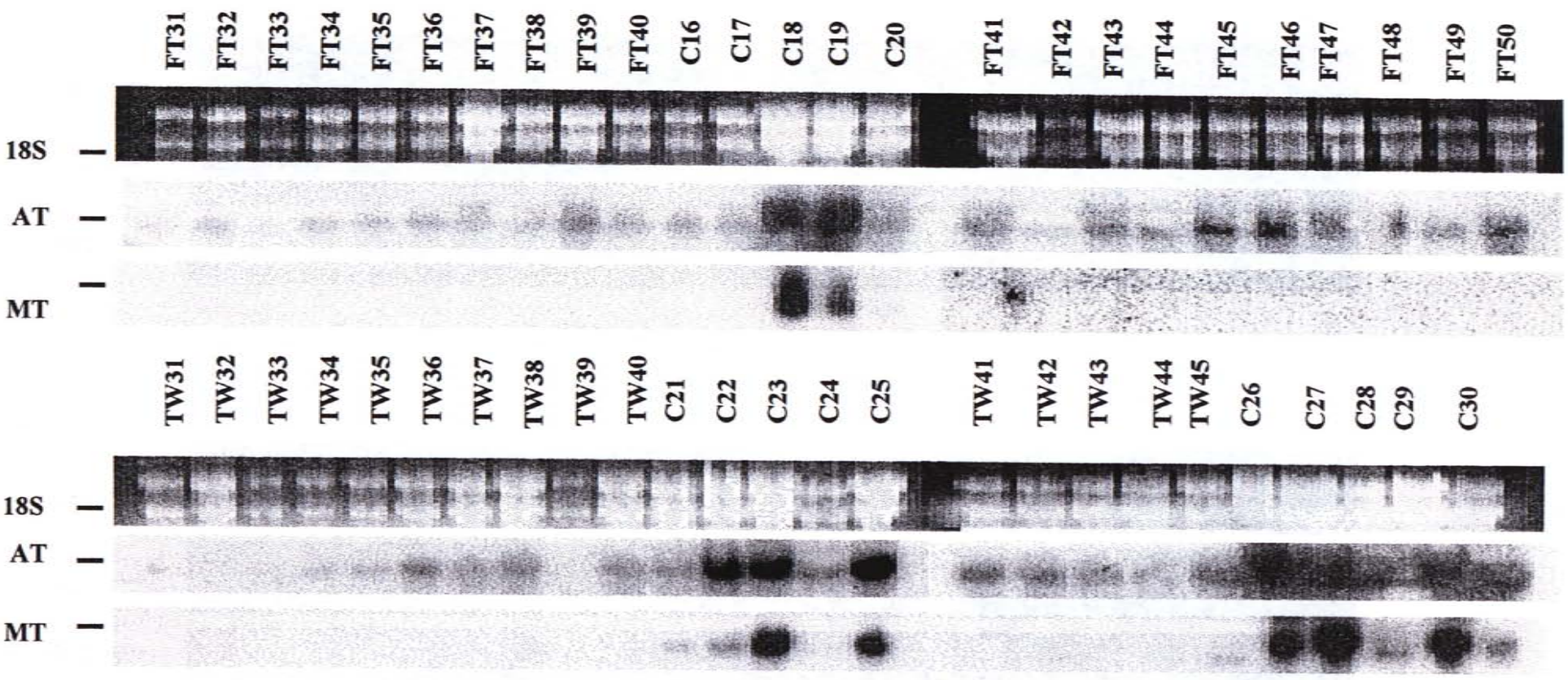


Fig. 4.5 Northern Blot Analysis of MT mRNA levels in the liver of tilapia from different sites, Tai Wai (TW) and Fo Tan (FT).

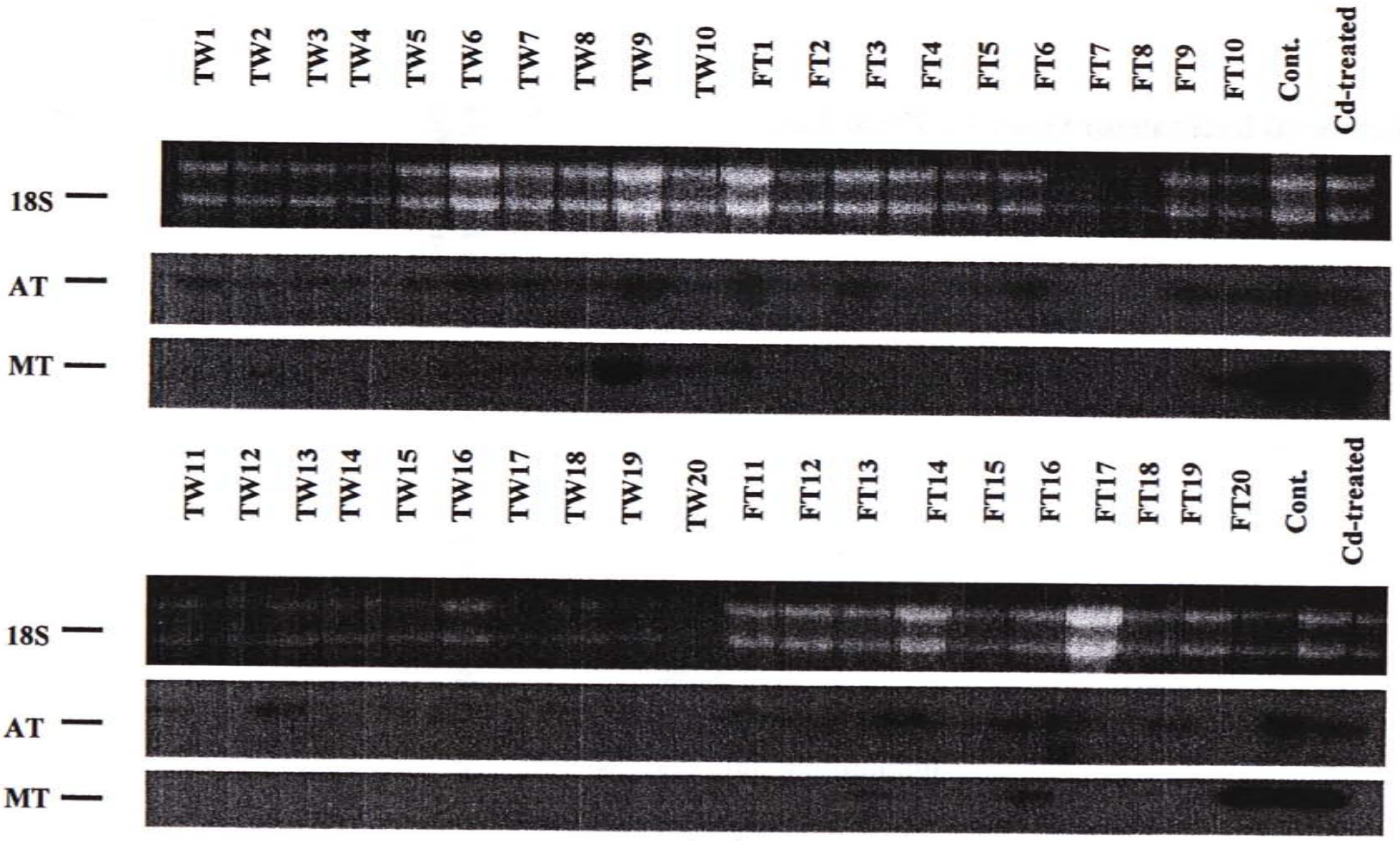


Fig. 4.6 Northern Blot Analysis of MT mRNA levels in the gills of tilapia from different sites, Tai Wai (TW) and Fo Tan (FT).

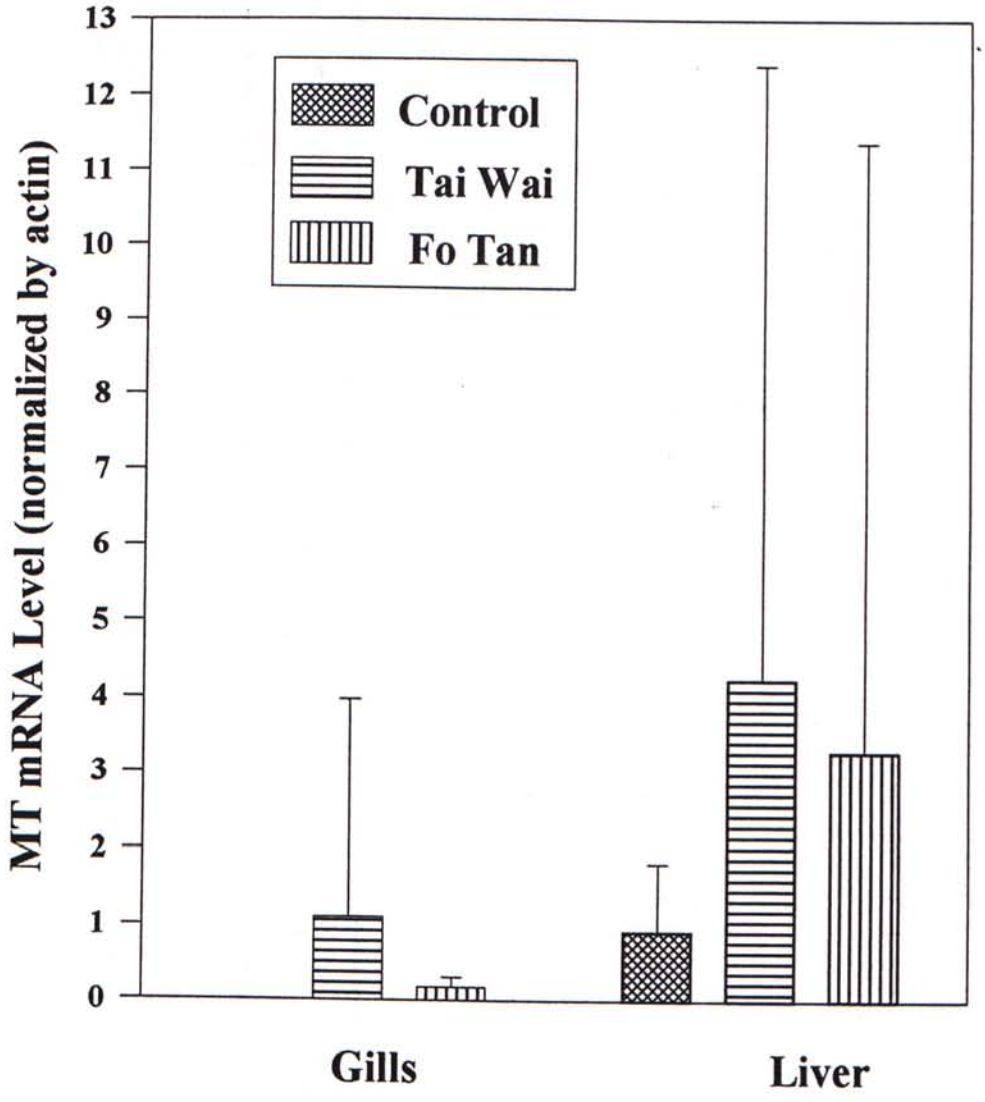


Fig.4.7. MT mRNA levels in different tissues of tilapia from different sites.

Fig 4.8a

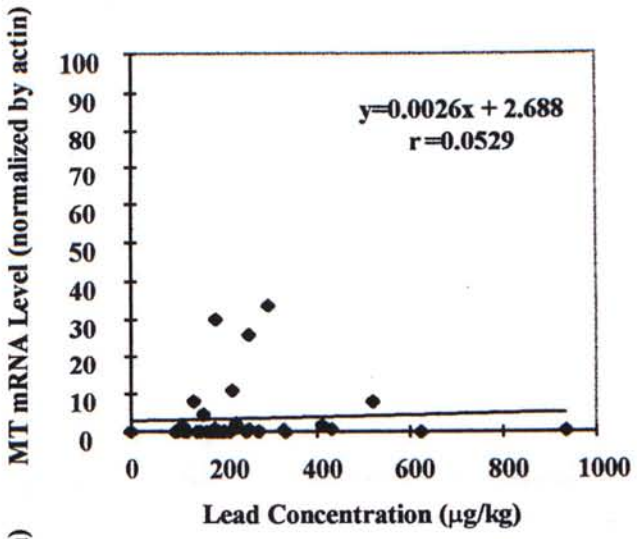


Fig 4.8b

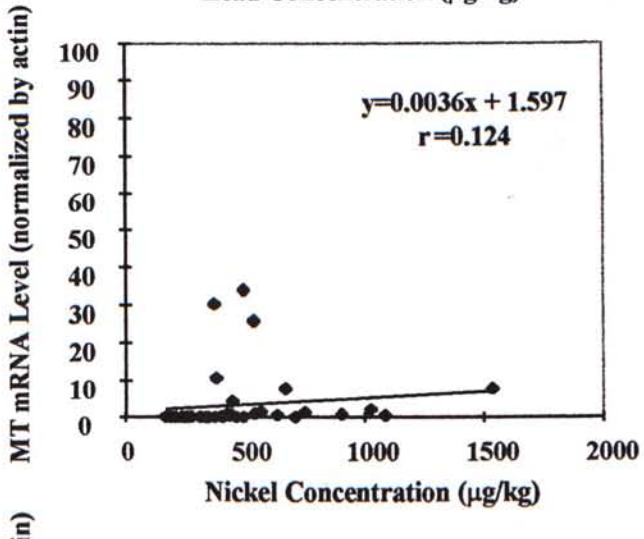


Fig 4.8c

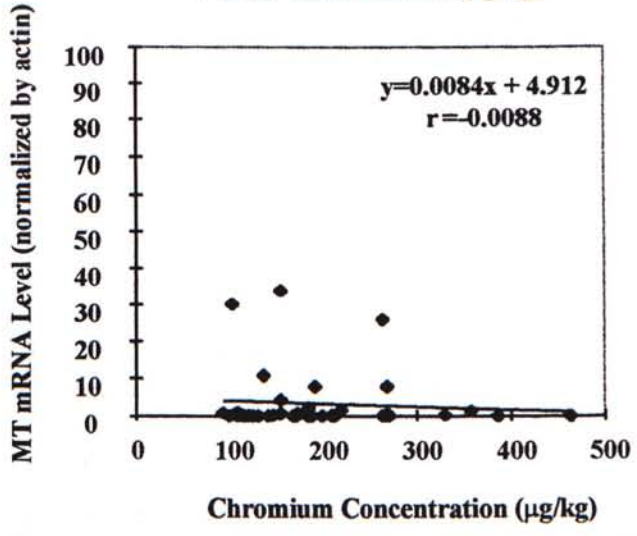


Fig. 4.8a, b and c. Assessment of relationship between MT mRNA level and lead,nickel or chromium concentration in the liver of feral tilapia from Fo Tan.

Fig 4.9a

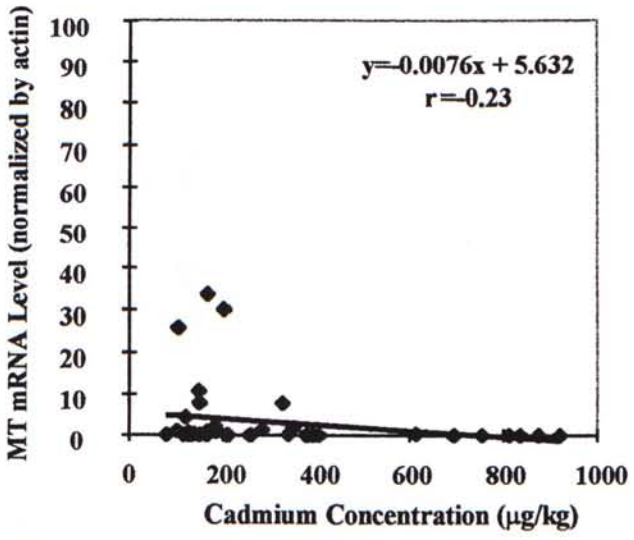


Fig 4.9b

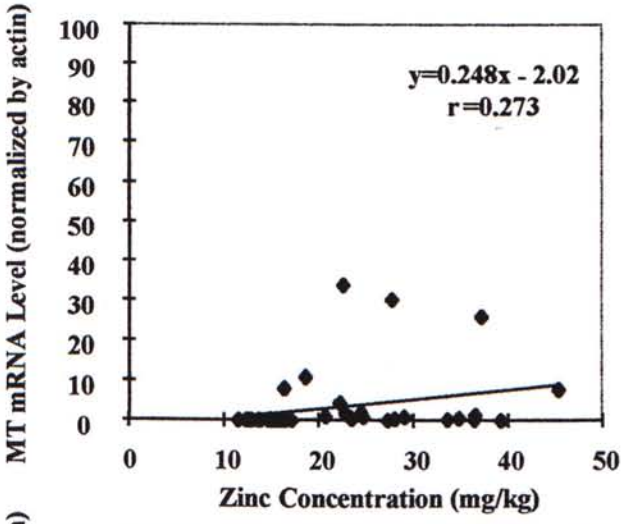


Fig 4.9c

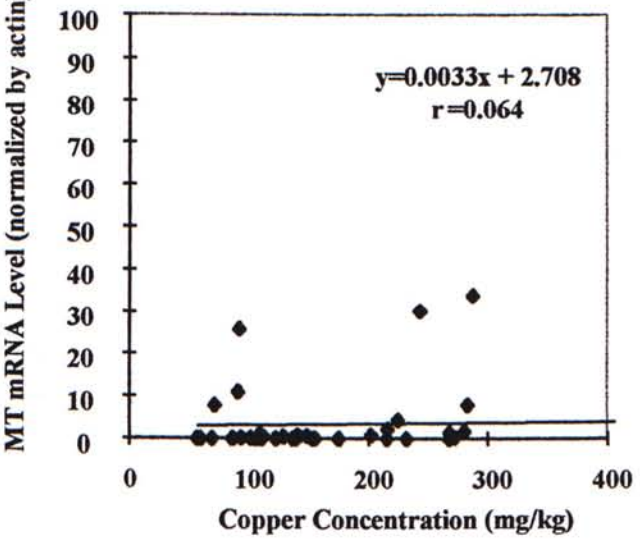


Fig. 4.9a, b and c. Assessment of relationship between MT mRNA level and cadmium, zinc and copper concentration in the liver of feral tilapia from Fo Tan.

Fig. 4.10a

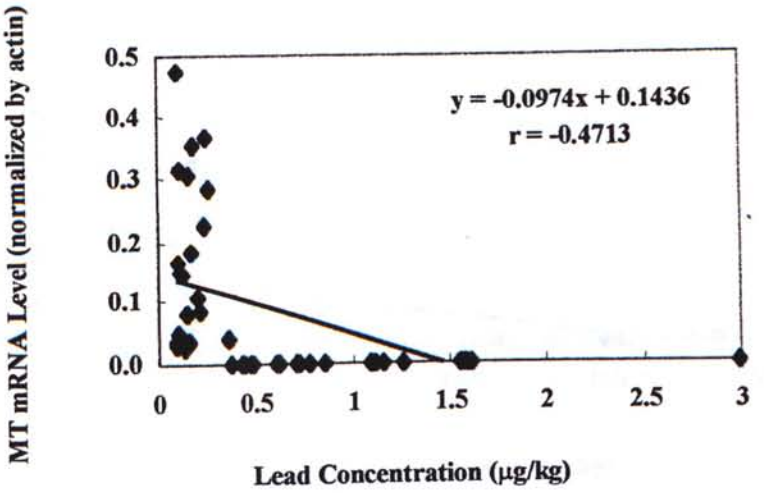


Fig. 4.10b

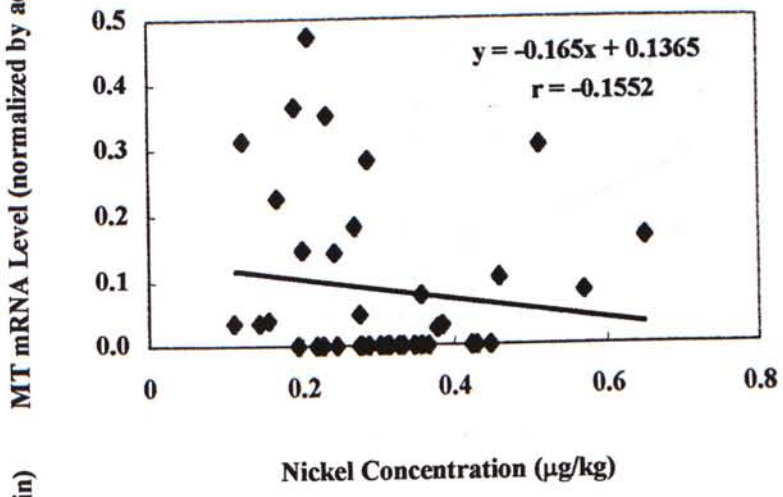


Fig. 4.10c

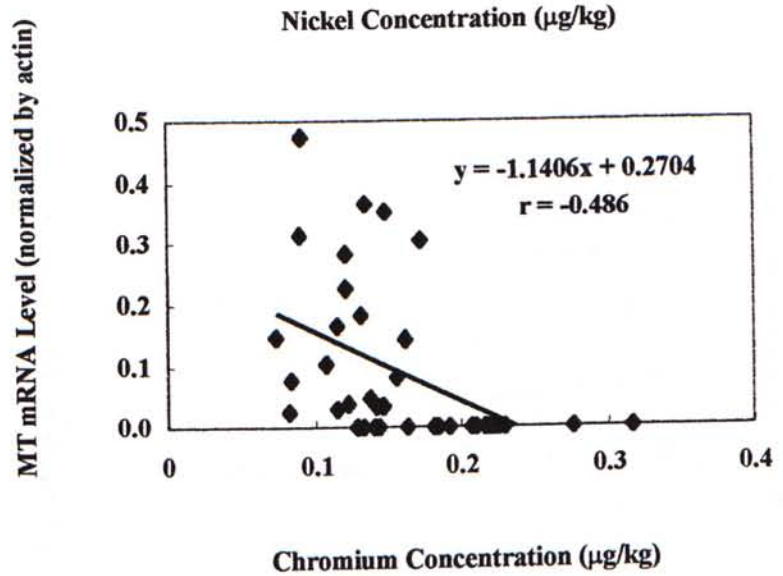


Fig. 4.10a, b and c. Assessment of relationship between MT mRNA level and lead, nickel or chromium concentration in the gills of feral tilapia from Fo Tan.

Fig. 4.11a

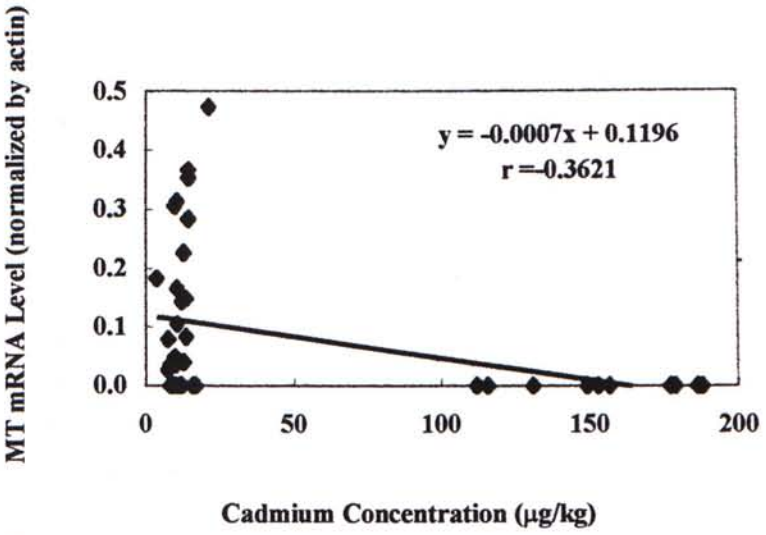


Fig. 4.11b

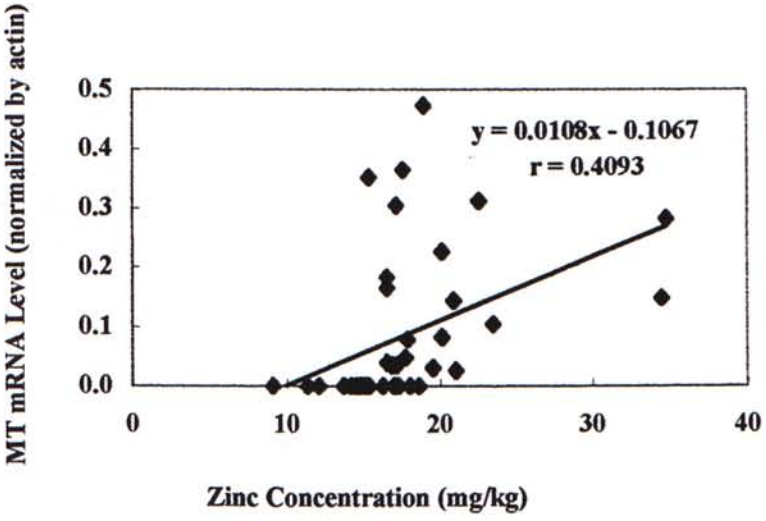


Fig. 4.11c

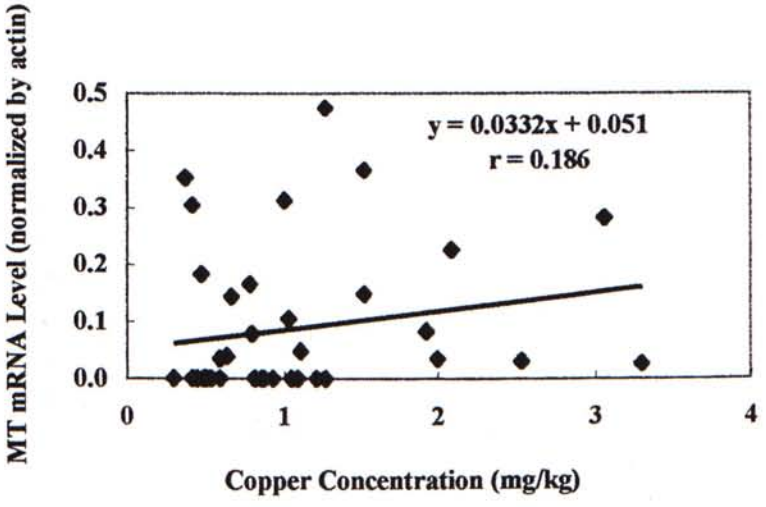


Fig. 4.11a, b and c. Assessment of relationship between MT mRNA level and cadmium, zinc or copper concentration in the gills of feral tilapia from Fo Tan.

Fig. 4.12a

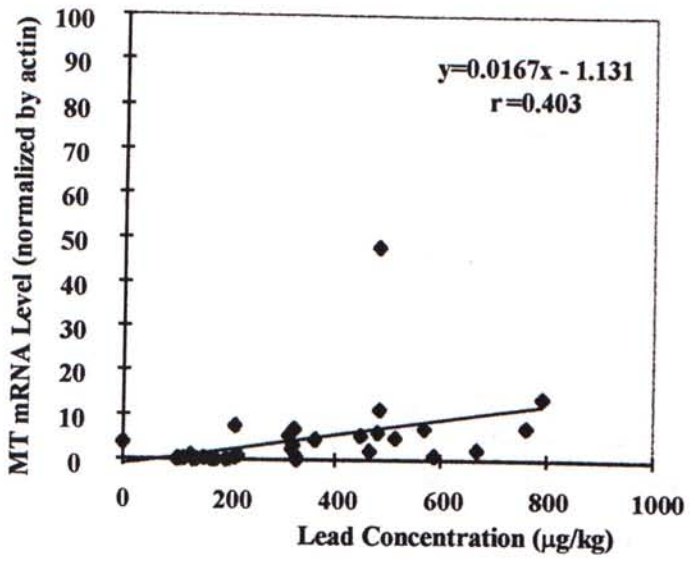


Fig. 4.12b

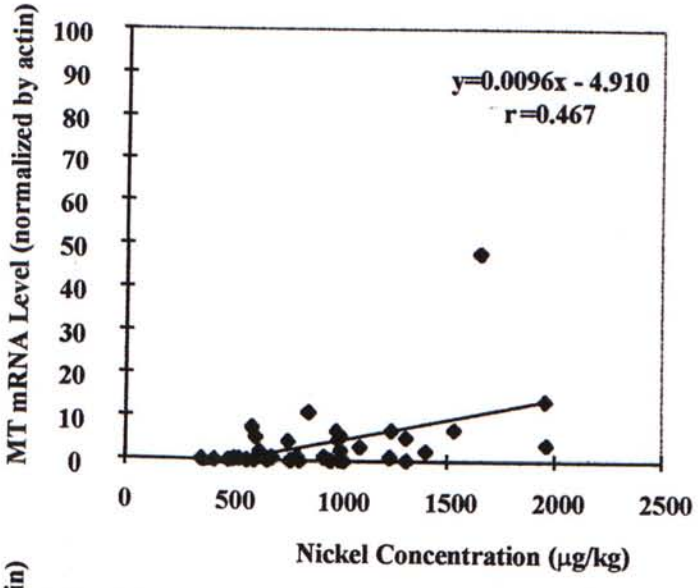


Fig. 4.12c

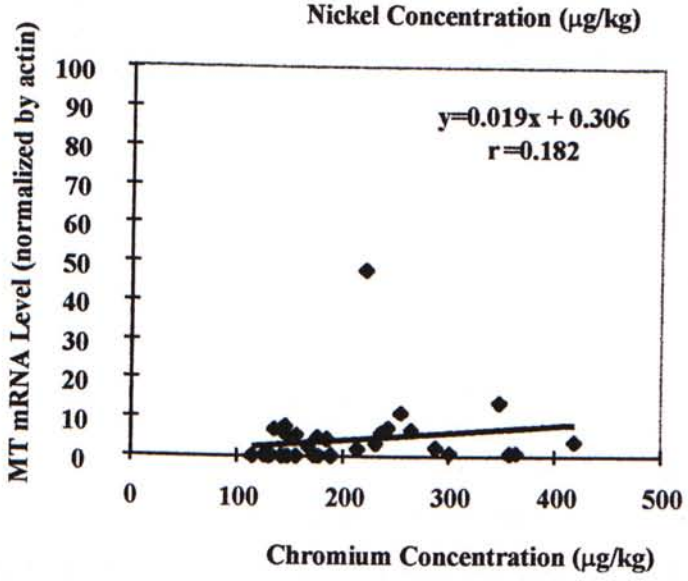


Fig. 4.12a, b and c. Assessment of relationship between MT mRNA level and lead, nickel or chromium concentration in the liver of feral tilapia from Tai Wai.

Fig. 4.13a

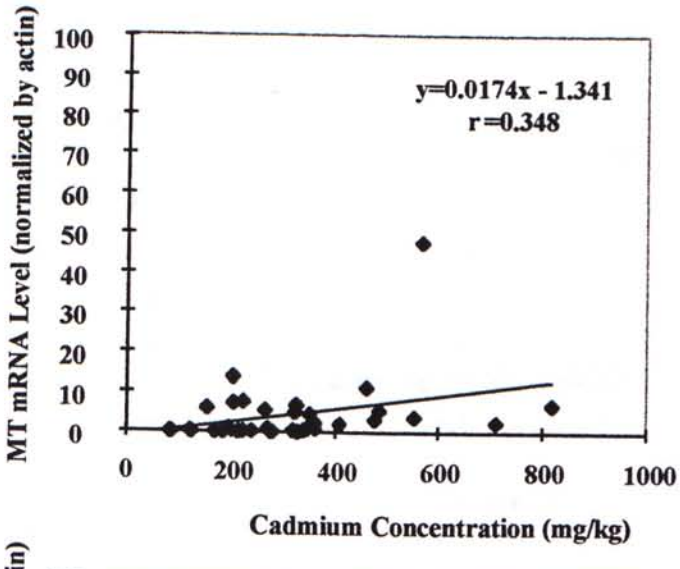


Fig. 4.13b

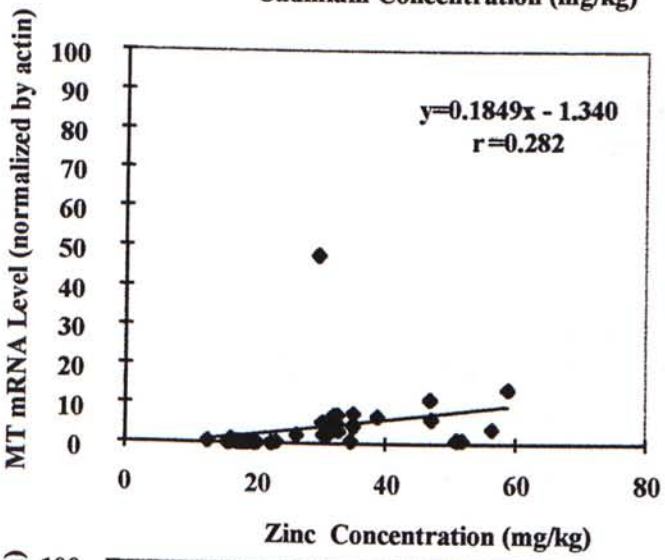


Fig. 4.13c

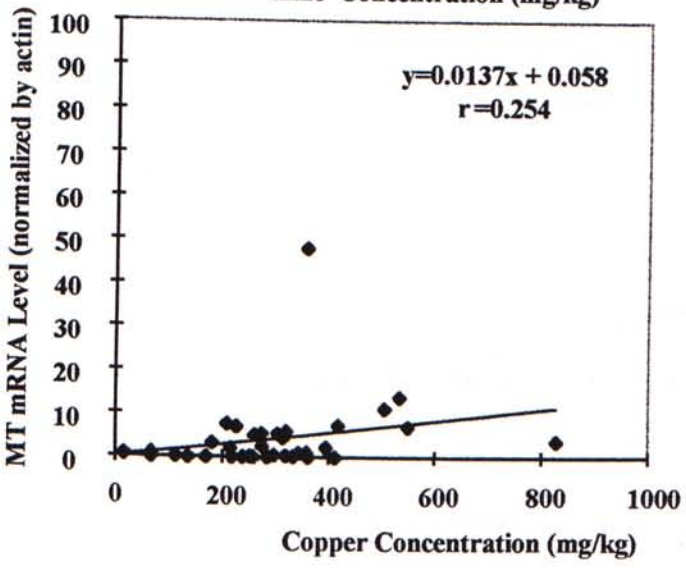


Fig. 4.13a, b and c. Assessment of relationship between MT mRNA level and cadmium, zinc and copper concentration in the liver of feral tilapia from Tai Wai.

Fig. 4.14a

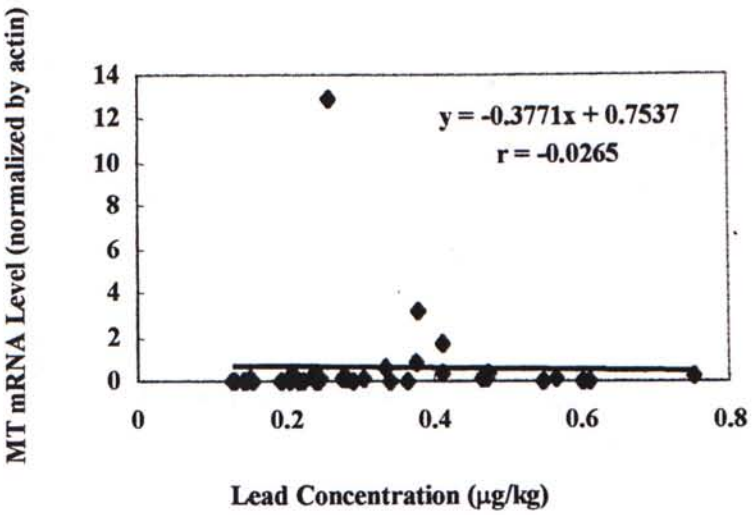


Fig. 4.14b

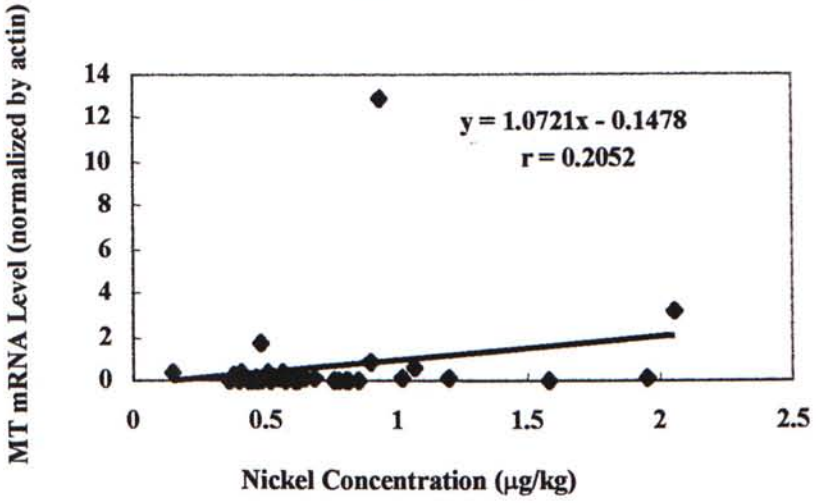


Fig. 4.14c

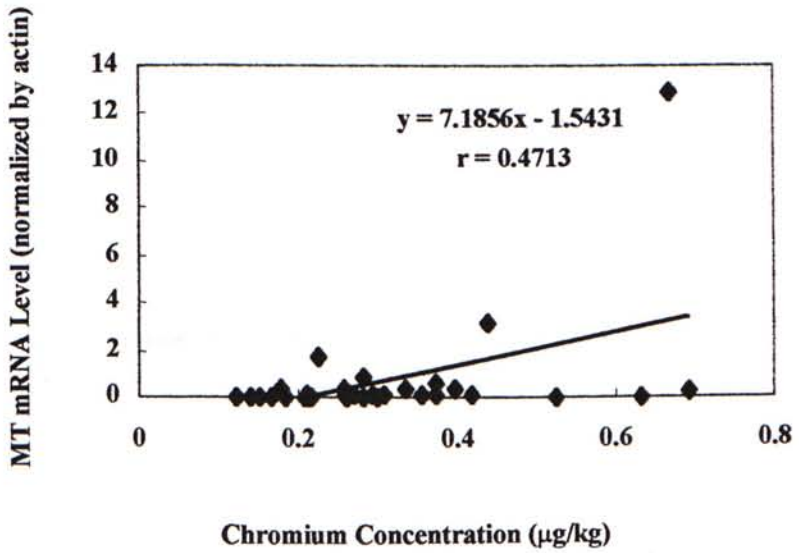


Fig. 4.14a, b and c. Assessment of relationship between MT mRNA level and lead, nickel or chromium concentration in the gills of feral tilapia from Tai Wai.

Fig. 4.15a

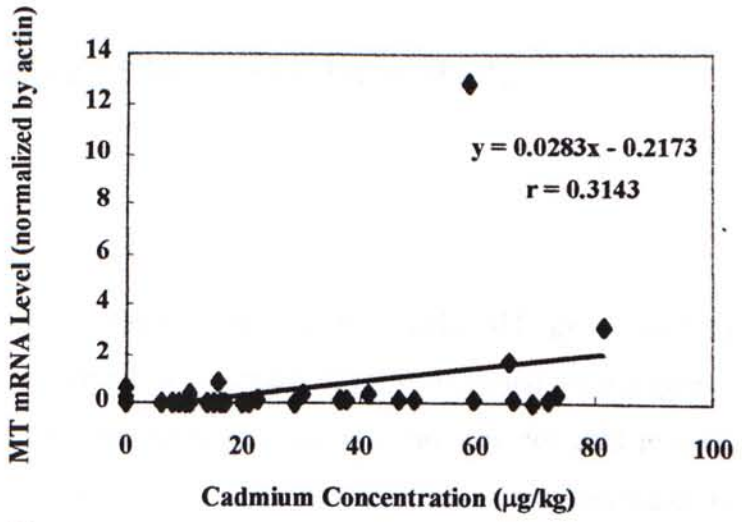


Fig. 4.15b

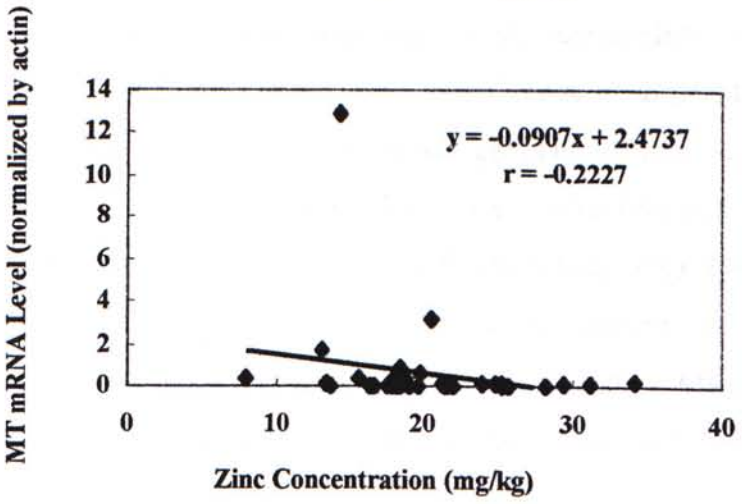


Fig. 4.15c

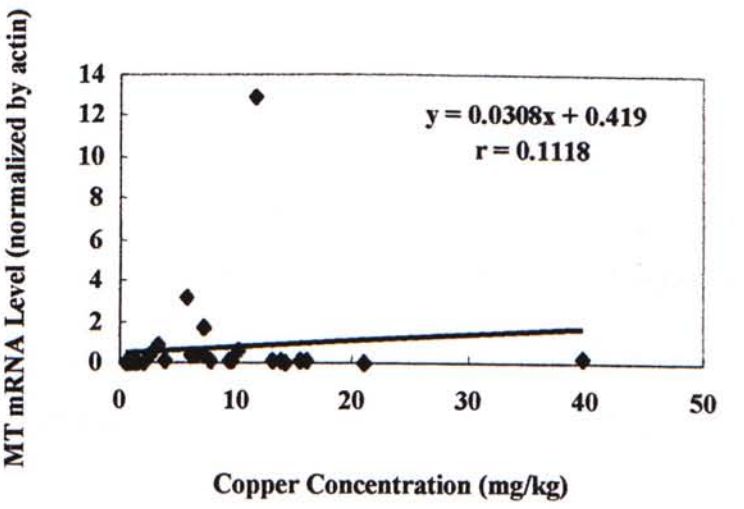


Fig. 4.15a, b and c. Assessment of relationship between MT mRNA level and cadmium, zinc or copper concentration in the gills of feral tilapia from Tai Wai.

Chapter 5 Cloning of Tilapia MT Genes

5.1 Introduction

Studies on the molecular biology of tilapia MT genes and their expression are also concerned. The number of isoforms found among species is quite variable. For instance, only one MT isoform was detected in marine flatfish such as plaice, turbot and winter flounder and some freshwater fish, stone loach and pike whereas common carp, eel, perch, scorpionfish and skipjack tuna were shown to have two MT isoforms (George *et al.*,1994). Apart from that, the sequences and the promoter of MT genes in fish were well studies such as rainbow trout MT-A (Olsson *et al.*,1995) and MTB (Zafarullah *et al.*,1988), pike and stone loach (Kille *et al.*,1993) and carp (Chan,1996). Fish MT genes and mammalian MT genes show some similar: (1) tripartite gene structure; (2) conservation of cysteine residues; (3) presence of multiple MREs in their 5' flanking regions. However, the MT gene in tilapia was never been investigated.

5.1.1 Specific Aims of this Chapter

In the present study, we study the structure of MT gene in tilapia using genomic PCR and southern blot analysis. The specific aims of this chapter are:

1. To determine the MT gene presented in the genome of tilapia.
2. To amplify the MT gene using PCR techniques and obtain the MT gene structure in tilapia.
3. To sequence the MT gene of tilapia.

5.2 Materials and Methods

5.2.1 Reagents

5.2.1.1 Preparation of Plasmid DNA

Solution I:

5 ml of 1M Tris-Cl, pH 8.0
4 ml of 0.5M EDTA
9 ml of 20% (w/v) glucose
2 ml of 10mg/ml RNase A
made up to 200 ml with ddH_2O

Solution II:

10 ml of 10% SDS
4 ml of 5M sodium hydroxide
made up to 100 ml with ddH_2O

Solution III:

0.6 ml of 5M potassium acetate
pH adjusted to 8.0 with glacial acetic acid
made up to 100 ml with ddH_2O

Tris-EDTA Buffer, pH 8.0 (TE buffer)

10 mM Tris-Cl, pH 8.0
1mM EDTA, pH 8.0

Ribonuclease A (RNase)

pancreatic RNase A (10mg/ml)
dissolved in 15mM NaCl/10mM Tris-Cl (pH 7.5)
heated up to 100°C for 15 minutes

5.2.1.2 Preparation of Genomic DNA

Grinding Buffer:

0.1M Tris
0.05M Na_2EDTA
0.2M NaCl
1% SDS

10M Ammonium Acetate:

770g ammonium acetate
made up to 1L with ddH_2O and sterilized by filtration

5.2.1.3 Restriction Enzyme Digestion

Gel loading buffer (6X):

40% (w/v) sucrose in water
0.25% (w/v) xylene cyanol
0.25% (w/v) bromophenol blue
made up to a final volume of 10 ml with ddH_2O

Tris-acetate-EDTA (TAE) buffer (50X):

242g of Tris-Cl
57.1 ml of Glacial acetic acid
20 ml 0.5M EDTA, pH 8.0
made up to 1 L with ddH_2O

1 % agarose gel for DNA

1 g agarose
100 ml of 1X TAE
Heat the mixture and pour the gel into gel tray after cooling
Wait until the gel polymerized

5.2.1.4 Vacuum Blotting of DNA (Southern Blotting)

Tris-boric acid-EDTA (TBE) buffer (5X):

54g of Tris-Cl
275g of Boric acid
20 ml of 0.5M EDTA, pH 8.0
made up to 1 L with ddH_2O

Denaturing Solution (for SSC transfer)

87.6 g NaCl
20.0 g NaOH
made up to 1 L with ddH_2O and filtered

Neutralization Solution (for SSC transfer)

121.1 g Tris
116 g NaCl
made up to 1 L with ddH_2O, pH adjusted to 5.0 and filtered

Standard Saline Citrate (SSC) (20X):

175.3g NaCl
88.2g Sodium Citrate
made up of 1L with ddH_2O, pH adjusted to 7.0 and autoclaved

5.2.1.5 Polymerase Chain Reaction

PCR Reaction Mixture (for Thermoprime^{Plus} DNA Polymerase)

2.5mM $MgCl_2$
1X Reaction buffer
1 unit Thermoprime^{Plus} DNA Polymerase
100 ng of each of the primer
2 µl DNA template
made up to 50 or 100µl with autoclaved ddH_2O

Deoxyribonucleotide (dNTP) mix (10mM):

20 µl 100mM dATP (Pharmacia)
20 µl 100mM dTTP (Pharmacia)
20 µl 100mM dCTP (Pharmacia)
20 µl 100mM dGTP (Pharmacia)
120 µl autoclaved ddH_2O

5.2.1.6 Transformation of *E.coli* Competent Cells

LB (Luria-Bertani) Medium

10 g bacto-tryptone
5 g bacto-yeast extract
10 g NaCl
made up to 1 L with ddH_2O and autoclaved

LB or CG plate

15 g agar
1 L CG (Bio101, U.S.A.) or LB medium and autoclaved

Isopropylthio-β-D-galactoside (IPTG)

2 g IPTG
added with 10 ml H_2O and sterilized by filtration
5-bromo-4-chloro-3-indoyl-β-D galactoside (X gal)
200 mg X gal
dissolved in 10 ml dimethylformamide and store at -20°C in dark

5.2.1.7 Nucleotide Sequence Determination (Maninatis, *et al.*,1982)

Tris-borate-EDTA buffer (TBE)

54 g Tris base
27.5g boric acid
20 ml 0.5 M of EDTA (pH 8.0)

Acrylamide Solution (20%): (Solution A)

96.5 g of Acrylamide

3.35 g of Methylene-bis-acrylamide
233.5 g of Urea
100 ml of 5X TBE
made up to 500 ml with ddH_2O and filtered

Urea Mix: (Solution B)
233.3 g of Urea
100 ml of 5X TBE
made up to 500 ml with ddH_2O and filtered

6 % acrylamide gel
(1) Casting tray sealing gel
6 ml 20 % Solution A
14 ml Solution B
250 µl 10% ammonium persulfate (APS)
100 µl N,N,N',N'-Tetra-methyl-ethylenediamine (TEMED)

(2) 50 ml casting gel
15 ml 20 % Solution A
35 ml Solution B
400 µl 10% APS
30 µl Temed

5.2.1.8 List of Primers

5.2.1.8.1 Primers used for Nucleotide Sequence Determination

Reverse primer: 5'AACAG CTATG ACCAT G 3'''
Universal primer: 5'GTAAA ACGAC GGCCA GT 3'''
T_7 promoter primer: 5'AATAC GACTA CTATA G 3'''
M_{13} reverse primer: 5'CAGGA AACAG CTATG ACCAT 3'''
AMTIIF1 primer: 5'CCAAG TAACA TGCAT AG 3''
AMTIIR1 primer: 5'AGAGC CACCT GCGCA CG 3''
AMTIR2 primer: 5'AGTTA AGTTT CATGC GGTC 3''
AMTIR3 primer: 5'CTTGT TAAAC AGCGG CAGAA CACTC 3'

5.2.1.8.2 Tilapia MT Specific Primers for PCR:

TMT5 primer: 5'' ATGGA TCCGT GCGAA TGCGC CAAG 3''
TMT3 primer: 5'' CACAA GTACA AAATC ATTCG TTAT C 3''

5.2.2 Methods

5.2.2.1 Preparation of Plasmid

For small scale plasmid preparation, the method of alkaline lysis described by Maniatis *et al.* (1982) was used. The pelleted bacterial cells were subsequently added with Solution I, Solution II and Solution III and the plasmids were precipitated in ethanol or isopropanol and then resuspended in autoclaved distilled water. The plasmid DNA prepared in this method is suitable for restriction enzyme digestion but not for DNA sequencing as RNA contamination might interfere sequencing reaction.

For large scale plasmid preparation, a commercial kit Magic™Maxipreps (Promega) was used. Alkali lysis as described above was used to lyse the cells to prepare crude plasmid DNA. RNA contamination is prevented by RNase treatment and the plasmid DNAs are subsequently absorbed on resin and eluted by preheated autoclaved distilled water. This method allows the purification of relatively large amount of plasmid DNA which is suitable for mapping of restriction enzyme sites, subcloning and DNA sequencing.

5.2.2.2 Preparation of Genomic DNA

Genomic DNA of tilapia was prepared according to the method as modified from Scott *et al.* (1985). In brief, muscle tissue (1 g) from tilapia was homogenized in 80 ml Grinding Buffer in the presence of liquid nitrogen with mortar. Proteins were removed by overnight proteinase K (Boehringer Mannheim) digestion followed by phenol-chloroform extractions. Genomic DNA was precipitated by adding 10M ammonium acetate (0.2 volume) and ethanol (2 volume). The precipitated DNA was washed by 70% ethanol and

dissolved in TE buffer. The purity and quantity of the genomic DNA was determined by the ratio of $O.D._{260}$ to $O.D._{280}$.

5.2.2.3 Restriction Enzyme Digestion

Restriction enzyme digestion of DNA was carried out in a volume of 10 to 200 µl reaction in a recommended buffer. The reaction mixtures were incubated at 37°C for at least 3 hours. The digested DNA fragments were mixed with gel loading and resolved on agarose gel in 1X TAE buffer.

5.2.2.4 Vacuum Blotting of genomic DNA (Southern Blotting)

Tilapia genomic DNA was digested with different restriction enzymes (10 units/µg genomic DNA) and the digested genomic DNA fragments are resolved on 0.7% agarose gel in 1X TBE buffer at 50V. Genomic DNA fragments were depurinated into smaller fragments with 0.2M HCl treatment (30 minutes) and 1.5M NaCl/1M Tris (pH 7.4) (30 minutes, twice), respectively and then transferred on to nylon membrane (Hybond N, Amersham) via vacuum transfer in 10X SSC. DNA fragments were fixed on the nylon membrane with UV crosslinking using UV Crosslinker (Fotodyne).

5.2.2.5 Radioactive Labeling of Nucleic Acid Probes

DNA fragments from agarose gels were purified using Sephaglas™ BandPrep Kit (Pharmacia). DNA fragments were labeled with α-^{32}P-dCTP (PB10205, Amersham) by using Nick Translation (N5000, Amersham). The unincorporated α-^{32}P-dCTP was removed by Sephadex G-50 spin-column chromatography (Maniatis *et al.*,1982).

5.2.2.6 Hybridization

Nylon membrane was pre-hybridized in 1X Hybridization Buffer (5X SSC, 1% SDS and 5X Denhardt's solution) at 65°C with the presence of calf thymus DNA (100 μg/μl) for 1 hour. Hybridization was carried out at 65°C for 16 hours in the same Hybridization Buffer with the denatured ^{32}P-labeled probes. Alternatively, nylon membrane was pre-hybridized with denatured ^{32}P-labeled probe for 2 hours. After hybridization, the membrane was washed with 2X SSC/0.1% SDS at room temperature once and at 65°C twice. Autoradiography was carried out with either Kodak XOmat-AR film or Kodak BioMax film.

5.2.2.7 Polymerase Chain Reaction

PTC-100 Thermal Cycler (MJ Research, USA) was used for performing PCR. Each of the 50 or 100 μl reaction mixture contained 1 unit Thermoprime^{Plus} DNA polymerase, 2 μl DNA templates, 100 ng each of the PCR primers, 0.2 mM deoxyribonucleotide (dNTP) (Pharmacia) in recommended buffer. The PCR conditions were 25 or 30 cycles of (1) 94°C, 1 minute; (2)55°C, 2 minutes; and (3) 72°C, 3 minutes. Any change in the PCR conditions would be specified. The template DNA were either cDNA or genomic DNA from juvenile tilapia (approximately 40ng/μl). PCR of the genomic DNA of tilapia was also performed using PCR Master Kit (Boehringer Mannheim).

5.2.3 Southern Blot Analysis of Tilapia Genomic DNA

Ten micrograms genomic DNA was digested with 100 units of *Bam*HI, *Eco*RI, *Hin*dIII or *Xba*I in recommended buffers (Section 5.2.2.3) for 4 hours or overnight until the digestion was completed. Digested genomic DNA fragments were resolved on 0.7% agarose gel in 1X TBE buffer and transferred on to nylon membrane via SSC transfer (Section 5.2.2.4). The membrane was

hybridized in Hybridization buffer with α-^{32}P-dCTP labeled full length MT cDNA of tilapia (Section 5.2.2.5 and 5.2.2.6) and autoradiograph was carried out at -70°C using Kodak X-Omat film with double intensifying screens for 96 hours.

5.2.4 Analysis of the Sequences of Tilapia MT Genes

5.2.4.1 Amplification of MT Genes using PCR

Specific primers for MT gene, TMT5 and TMT3 were designed from the 5' end and 3' untranslated region of tilapia cDNA respectively (Chan, 1994). These primers were used to amplify the MT gene(s) of tilapia by using PCR master kits (Boehringer Mannheim). Thirty cycles PCR were carried out at denaturing temperature at 94°C for 1 minute, annealing temperature at 55°C for 2 minutes and extension temperature at 72°C for 3 minutes. The PCR products were resolved on 2% agarose gel in 1X TAE buffer.

5.2.4.2 Cloning of the MT Genes

For cloning of PCR products, the PCR products were purified by Sephaglas™BandPreq Kit (Pharmacia) and then ligated into PCR™II vector (Invitrogen) using TA cloning ® Kit.

5.2.4.3 Transformation of *E.coli* Competent Cell

Either JM109 or XL-1 Blue competent cells were used for transformation of plasmid vectors. Ice thawed competent cells (100 µl) were mixed with plasmid DNA containing desired insert and then kept on ice for 30 minutes. The bacterial cells were subjected to a heat shock at 42°C for 90 seconds and then quickly chilled on ice for 2 minutes. The bacterial cells were recovered in 1 ml CG or LB medium at 37°C for 45 minutes. Ten µl to 1 ml of

bacterial cells were spread on a CG or LB agar plate containing ampicillin, 20 µl 100 mM IPTG and 20µl 2% (w/v) X-gal as selection medium. The bacteria of blue colonies contain plasmid without DNA insert whereas white colonies usually have. White colonies would be selected for plasmid preparation, confirmation of insert size and/ or DNA sequencing.

5.2.4.4 Nucleotide Sequence Determination

The sequencing reactions were performed using the dideoxynucleotide chain termination method with the T7 DNA Sequencing Kit (Pharmacia). Plasmid DNA template of 2 to 10 µg was used for the sequencing reactions. The sequencing products were resolved on either a 6% or 8% denaturing polyacrylamide gel. The polyacrylamide gels were lifted on to a 3 MM paper and dried under vacuum suction at 80°C for 2 hours until the gel was dried. Either Kodak X-Omat or Kodak Biomax film was used for autoradiography.

5.3 Results

5.3.1 Southern Blot Analysis of Tilapia Genomic DNA

The result of southern blot analysis was shown in Fig. 5.2. Two hybridization signals with different intensity were observed in B*am*HI, E*co*RI and H*in*dIII-digested genomic DNA whereas in X*ba*I-digested genomic DNA, 3 signals were appeared. There was a stronger signal with about 9k bp in E*co*RI-digested genomic DNA. Furthermore, a signal with less than 2.5 kbp were obtained in H*in*dIII and X*ba*I digested genomic DNA.

5.3.2 Amplification of MT Gene Fragments Using PCR

From the Fig.5.3a, it indicated the genomic PCR products using different primers. O9, SG and NS primers were designed from satellite DNA

and they were act as positive control. But 09 primer and NS primers, which ould not shown any signal in the gel. In lane 2,8 and 14, the positive control using SG primers showed similar pattern in genomic DNA from different sites. By using MT primers and templates from different sites, a sharp signal with 600bp was obtained in lane 4, 10 and 16. In the Fig.5.3b, only genomic PCR products using MT primers could be hybridized by MT cDNA probe and consequently, a dark band was showed in lane 4, 10 and 16 simultaneously.

5.3.3 Analysis of the Sequences of Tilapia MT Genes

Genomic PCR products were performed using genomic DNA from different sites and only exons, introns and 3' untranslated region were sequenced. According to the Fig.5.4, the sequences were quite similar. They possessed three exons (25, 66 and 92 bp for exon 1, 2 and 3 respectively) interrupted by 2 introns (92 for intron 1 and 238 bp for intron 2). There was 103 bp in 3' untranslated region of tilapia MT. They are highly homologous from different sites (97%). The position of the introns at amino acid position 9 and 31 were found. The molecular size of the genes including exons, introns and 3' untranslated regions was at least 610 bp.

Comparing with the tilapia from AFD, MSL and Fo Tan, substitutions of nucleotides are found only in the introns. There are 13 and 18 substitutions of nucleotides in the tilapia from MSL and Fo Tan respectively, out of the 617 nucleotides aligned.

5.4 Discussion

5.4.1 Fish MT Genes

The regulation of MT gene is mostly concentrated in 5'-untranslated regions. The 5'-untranslated regions also call promoter regions, which possess several regulatory elements. One of them is metal responsive element (MRE)

with a 9-bp core sequence in mouse (TGCRCNCGG) (Stuart *et al.*,1985) which regulate the MT expression either in normal conditions or against metal exposures. The presence of MRE is quite variable among species (Olsson *et al.*,1997). For examples, up to 6 MREs were identified in rainbow trout but fewer MREs were located in pike (Kille *et al.*,1993, Olsson *et al.*,1997) and as a result, the pike MT gene is less inducible than rainbow trout (Olsson *et al.*,1997). In rainbow trout, these 6 MREs were organised into 2 clusters, one containing two copies of MREs at the proximal region of TATA box sequence and the remained MREs are located at the distal end from the start of transcription (Olsson *et al.*,1995). The activation potentials of MREs are different against metals. Stuart *et al.* (1985) reported that MREd in mouse MTI promoter is the strongest inducible element. In the present study, only structure and 3'-untranslated regions in tilapia caught from different sites were cloned and sequenced. However, the promoter regions in tilapia MT genes are still obscure.

By southern blot analysis of tilapia (MSL) genomic DNA, two (B*am*HI, E*co*RI and H*ind*III-digested) or three bands (X*ba*I-digested) were observed in Fig.5.2 suggesting the existence of at least 2 MT genes which matched the results described previously in rainbow trout (Zafarullah *et al.*,1988; Bonham *et al.*,1987). Kito *et al.* (1986) reported that 2 forms of MT proteins with different amino acid composition were identified in the kidney and liver of common carp. However, this is still obscure whether more than one MT isoform are exist in tilapia. In PCR analysis, only one genomic fragment was shown in Fig. 5.3. However, the number of PCR clones being sequenced was limited and if more clones were sequenced variants could be found. The amplified MT gene fragment shares similar properties to other MT genes of the teleost species. They had tripartite sturcture and the codon for serine residue which was the boundary residue between α and β domains, was separated by the second and third exons. The amino acid sequences, which determined from nucleotide sequence of tilapia, indicated that they were the same as the tilapia MT amino

acids sequence from tilapia cDNA (Chan,1994). Chan (1994) presented that the tilapia MT showed 83% amino acid sequence identity with the flounder MT and their locations of cysteines were highly conserved in fish MTs in Fig.5.5.

5.5 Conclusion

In the present study, the MT gene fragments were tripartite which 3 exons were interrupted by 2 introns and there were quite similar in MT gene sequence of tilapia from different sites.

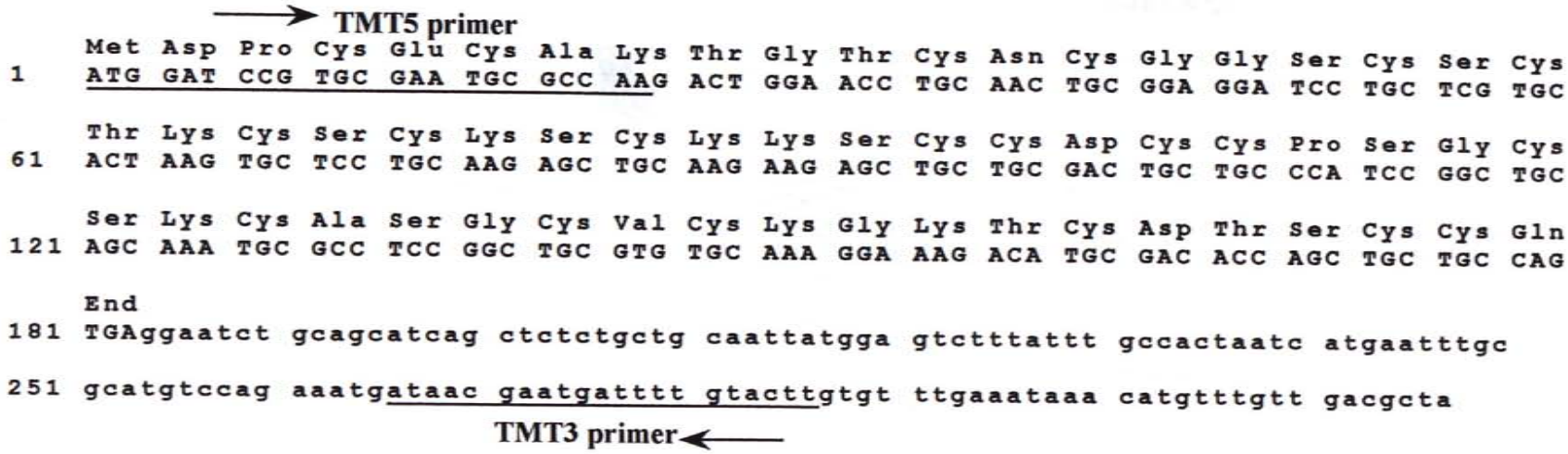


Fig.5.1 Nucleotide Sequence of tilapia (*Tilapia mossambica*) Metallothionein cDNA (Chan, 1994). The complete coding region and the predicted amino acid sequence of a cloned MT cDNA are shown. Lower-case letters are used to denote the untranslated region; the last "a" represents the first of 17A residues.

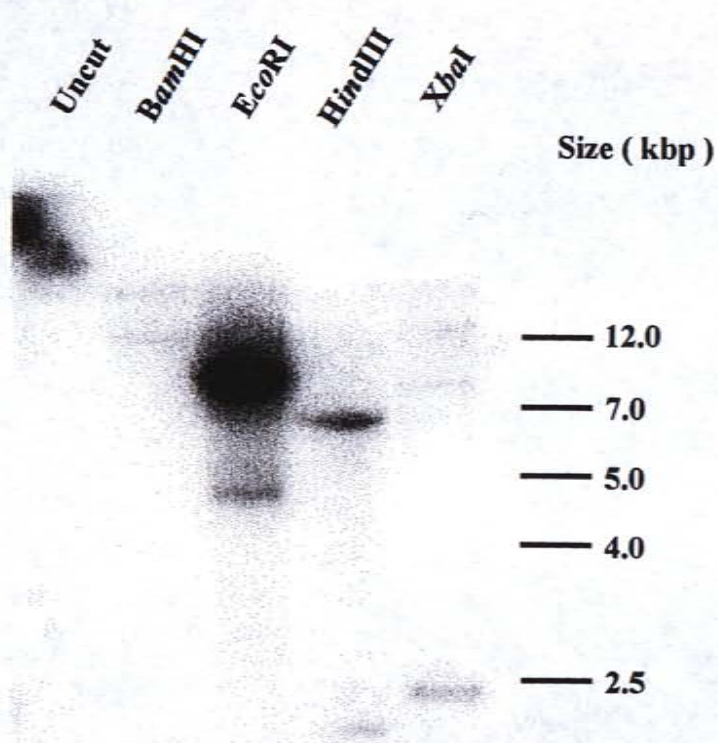


Fig.5.2 Southern Blot Analysis of Tilapia Genomic DNA with full length MT cDNA of *Tilapia niloticus*.

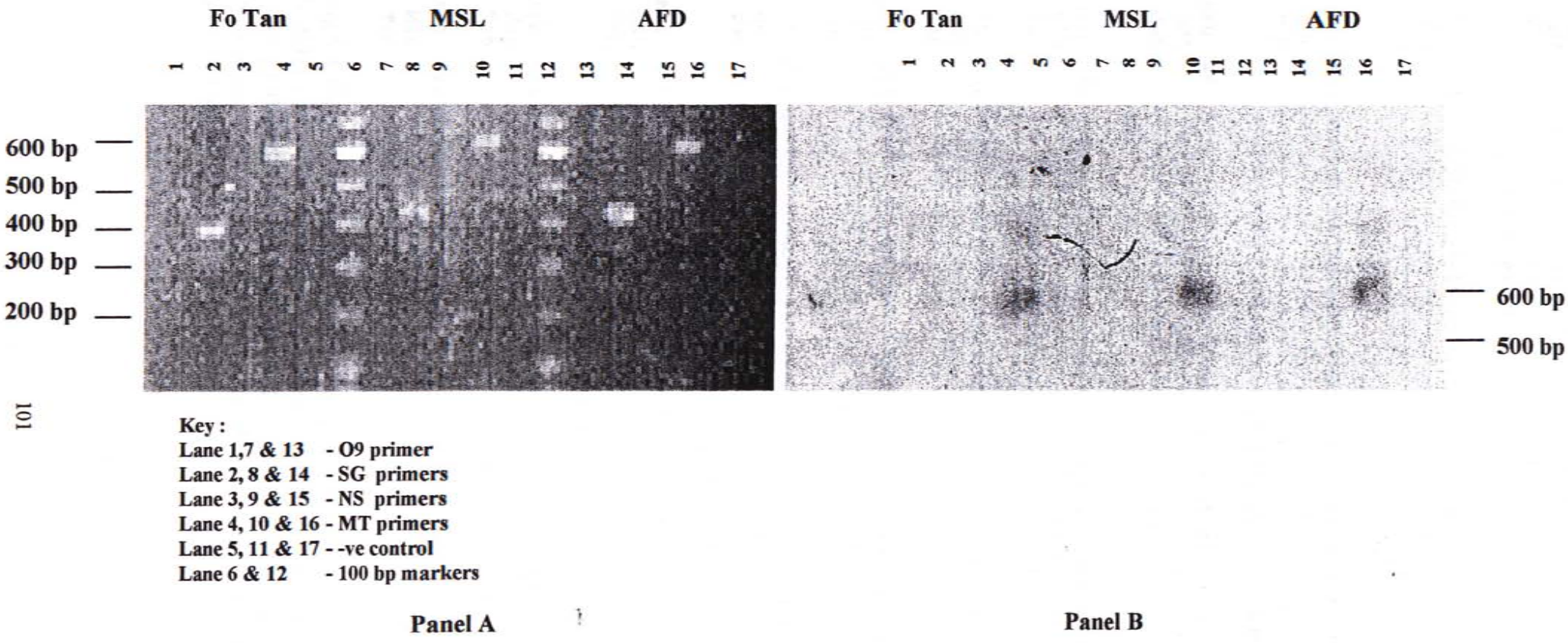


Fig. 5.3 PCR Analysis of Tilapia MT Genes from AFD(*Tilapia mossambicus* x *aurea*), MSL(*Tilapia niloticus*) and Fo Tan (*Tilapia mossambicus*). Panel A Genomic PCR products were visualized in 2% agarose gel . **Panel B** Southern Blot hybridized with full length MT cDNA of *Tilapia niloticus*.

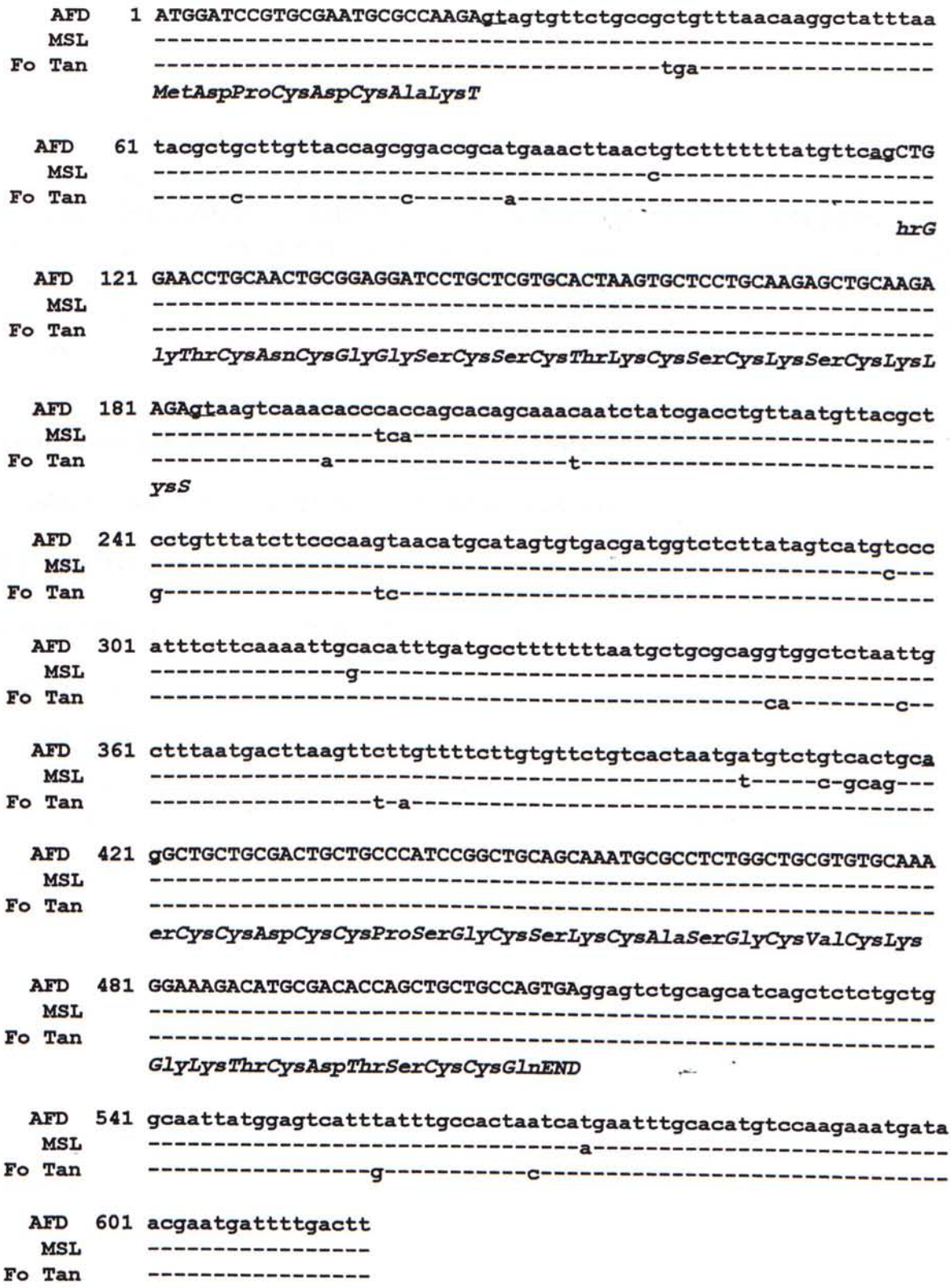


Fig.5.4 Nucleotide Sequences of Tilapia MT Genomic DNA Fragment. The bold nucleotides mark the exons of the amplified MT gene fragment and the amino acid sequence predicted from the amplified MT gene fragment is indicated in italic letters. The underlined sequences show the primer annealing sites. The splicing junctions (gt-ag) and the stop codon (END) are also marked.

```
10         20         30         40         50         60
MDPCECSKTGTCNCGGSCTCKNCSCTTCNK SCCPCCPSGCPKCASGCVCKGKTCDTTCCQ (1)
----------S-------K-S--A--S-K-A---D------S---------------S--- (2)
----------S-------K-S--A--S-K- --------D-S---------------S--- (3)
---------------AT-K-T-------K- ---F------S--------N-NS-GSS--- (4)
------A-----------S-TK---KS-K- ---D------S---------------S--- (5)
```

(1)Winter Flounder MT, adapted from Chan *et al.*, 1989.

(2) Rainbow Trout MT-A, adapted from Bonham *et al.*, 1987.

(3) Rainbow Trout MT-B, adapted from Bonham *et al.*, 1987.

(4) Stone-Loach MT, adapted from Kille *et al.*, 1991.

(5) Tilapia MT, adapted from Chan, 1994.

Fig.5.5 Alignment of fish MT Amino Acid Sequences. Metallothioneins are highly conserved in Teleosts and the 20 Cysteine Residues Remain Unchanged.

Chapter 6 General Conclusions

In the present study, the induction of MT gene in the tissues of tilapia exposed to metal administration was studied. MT mRNA was constitutively expressed in different tissues of tilapia and some of the tissues possessed the endogenous levels of MT probably for maintaining normal metabolism. Liver is the tissue which showed high MT mRNA levels after metal injections, and is thus an important organ for metal detoxification, but the actual mechanism is still obscure. However, as the fishes were treated with i.p. injections of metals, which is not a natural way of pollutant uptake into the body of fish, aqueous exposure experiments were used to test the induction of MT mRNA in tilapia. In the aqueous exposure experiments, gills gave higher fold induction of MT mRNA than liver of tilapia exposed to lower doses of metals. It is believed that in aqueous exposure, gills is one of the primary organs to transfer metal ions to other internal organs. In conclusion, the MT gene expression in tilapia is tissue specific, route specific and metal specific.

In field study, no correlations between metal concentrations and MT mRNA levels in the tissues were obtained. This is because in the northern blot analysis, the endogenous MT mRNA levels in the tissues (gills and liver) of tilapia from Shing Mun River are not high enough to give significant signals and therefore, the MT mRNA levels in the tissues of tilapia from Shing Mun River might be under-estimated using northern blot analysis. RT-PCR is the alternative, which is very sensitive to measure the trace amount of MT mRNA levels by amplification of MT mRNA (Schlenk *et al.*,1997). Although metal concentrations have no significant correlation with MT mRNA, MT might be the alternative factor, which can be determined by Enzyme-linked Immunosorbent Assay (ELISA) or Radioimunoassay (RIA) for monitoring purposes. Hogstrand *et al.* (1990) reported that the induction of hepatic MT protein in blue-striped grunt after cadmium injection was detected using RIA,

and MT was found to be environmentally induced in blue-striped grunt and tomtate, caught at heavy-metal polluted areas.

In fact, waterborne metal concentrations and sediment metal concentrations have not been included in the present study. According to the report from Lee (1997), the metals concentrations such as zinc in water samples and sediment in Shing Mun River were highly correlated with metal concentrations in the tissues such as viscera of tilapia caught from Shing Mun River (r=0.9998,0.5848 respectively). It is believed that the MT protein or MT mRNA levels might reflect waterborne and sediment metal levels.

In this study, the southren blot analyses of the genomic DNA of tilapia supported that at least one MT genes in its genome. Specific primers were designed from the tilapia cDNA (Chan,1994) to amplify the MT genes using PCR techniques. These MT genes from different sites were cloned, sequenced and determined their genomic organization. Sequencing of three MT genes tilapia obtained from AFD, Fo Tan and MSL revealed that they were highly homologous (about 97%) and showed tripartite structure which possessed three exons interrupted by 2 introns. The 3' untranslated regions of MT genes in tilapia from different sites were quite similar whereas the 5' untranslated and further upstream regions of MT genes in tilapia have not be identified yet and therefore, the regulation of MT genes in tilapia are still unknown.

In summary, at least one MT gene was presented in tilapia genome using southern blot and PCR analyses. The MT genes from different sites showed highly homology including the structure gene and 3'-untranslated region. Intestine showed the highest endogenous level of MT mRNA and liver with lesser extent. Cadmium, copper and zinc induced MT mRNA expression in different tissues especially in liver and kidney in some cases, which showed the highest fold induction of MT mRNA. In zinc-treated carp, kidney gave higher induction MT mRNA levels than other tissues. From the result of aqueous

exposure experiments, there was positive correlation between fold induction of MT mRNA and time course at lower doses of cadmium and zinc. Comparing with gills, liver showed lower fold induction of MT mRNA at 10 μg/L.

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